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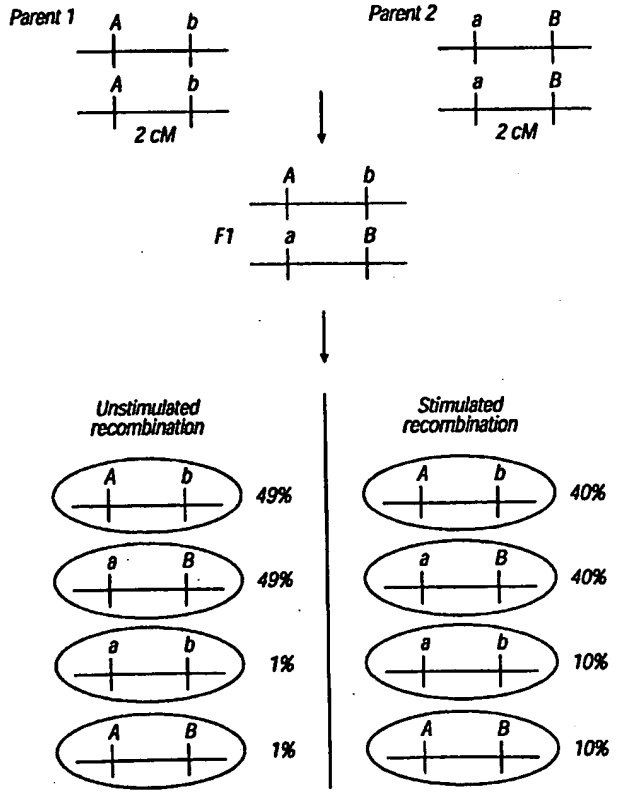
(54) Title: CHEMICAL AND PHYSICAL TREATMENT THAT STIMULATE RECOMBINATION

(57) Abstract

Disclosed are a number of physical or chemical treatments that increase the frequency of recombination in a plant. These treatments act to either cause DNA damage or modifications, affect chromatin structure, or affect the recombination machinery in the cell. Increases in the frequency of recombination are useful for improving a number of manipulations with plants including traditional plant breeding to form hybrids, self-pollinating plants in order to eliminate undesirable traits, introgression of a single trait from one plant line into another, generating novel mutations with novel desired phenotypes, genetic mapping experiments, and generation of transgenic plants by transformation with exogenous genetic material. Both homologous and non-homologous recombination frequencies are increased, as well as an increased frequency of recombination during meiosis and mitosis.



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DESCRIPTION

CHEMICAL AND PHYSICAL TREATMENTS THAT STIMULATE RECOMBINATION

BACKGROUND OF THE INVENTION

The government owns rights in the present invention pursuant to U.S. Department of Agriculture Grant No. 96-35304-3491, National Science Foundation Grant Nos. 9872641 and 9513511 and Grant No. DOEDE-FG05-920R22072 from the Consortium for Plant Biotechnology.

I. Field of the Invention

The present invention relates generally to the field of molecular biology. More particularly, it concerns methods that stimulate recombination in plants.

II. Description of Related Art

Recombination results in new arrangements of genes by various mechanisms such as assortment and segregation, crossing over, gene conversion, transformation, conjugation, transduction, or mixed infection of viruses. Recombination in plants can occur at numerous stages of plant development as well as numerous stages of plant manipulation. Any situation where the desired result is a new arrangement of genes is brought about by recombination. Examples include traditional plant breeding to form hybrids, self-pollinating plants in order to eliminate undesirable traits, introgression of a single trait from one plant line into another, generating novel mutations with novel desired phenotypes, genetic mapping experiments, and generation of transgenic plants by transformation with exogenous genetic material. Recombination events can proceed via homologous or non-homologous recombination.

In general, these recombination events occur at low frequencies, necessitating examination or screening of large numbers of candidate progeny to isolate a plant with the desired genetic event. For example, if a breeder wants to produce plants that

contain genes from two different parents, examination of thousands of progeny in order to find the correct genetic combination is often times necessary.

Another example of the use of recombination involves moving one desired trait from one plant line into another, a process called introgression. This involves the initial cross between two different plants, followed by many backcrosses of selected progeny plants to one of the parents in order to isolate the desired trait in the new background, all of which are dependent on the rates of recombination.

Another example of the use of recombination is genetic mapping. This process is dependent on generating novel, non-parental combinations of markers which is often times difficult to accomplish in regions of low recombination frequency, such as the centromeres, or when markers are tightly physically linked.

Yet another example of the use of recombination is the generation of transgenic plants via delivery of exogenous DNA into plants. Use of *Agrobacterium* mediated transformation, particle bombardment, liposome fusion, electroporation and so forth result in generating transgenic plants generally through non-homologous recombination events. Homologous recombination, while well established in yeast, bacterial and mammalian systems, has been much more difficult to accomplish in plants, partly due to low recombination frequencies.

Finally, generation of novel plant mutants with desired phenotypes is dependent on recombination frequencies. The range of mutations may be as simple as single base pair deletions, insertions or substitutions, more complicated such as large gene deletions or duplications, or extensive such as chromosomal breakage and translocations. Isolating these mutations in the desired genetic background and making these mutations permanent in a plants genome is dependent upon the frequency of recombination.

A number of parameters, in principle, effect recombination frequency. These can be grouped into at least three broad areas. The first general area that effects recombination frequency is DNA damage, such as strand breakage, base substitutions and deletions, as well as DNA modifications, such as methylation, crosslinking and adduct formation. DNA damage and modifications activate the DNA repair and recombination machinery of the cell. The second general area is bringing about a change in chromatin structure or the conformation of DNA in an organism's genome. Open conformation is associated with actively transcribed regions of DNA, regions of open chromatin or nucleosome structure (euchromatin), and during DNA replication and cell division. Thus it is thought that the more "open" the conformation of a given region of DNA, the more accessible that region is to recombination. Chromatin structure also can be effected by the degree of acetylation of histones or by treatments or agents that directly bind and/or alter DNA confirmation. The third general area that effects recombination frequency relates to the proteins of recombination machinery. Examples include the absolute level of recombinases or the state of activation of recombinases present in a cell, changes that effect the activity or specificity of recombinases, changes in ancillary proteins that interact with recombinases, and transgenic recombinases or ancillary proteins.

Here, the inventors describe chemical and physical treatments that cause or affect DNA damage and modifications including, but not limited to, U.V. exposure, methanesulfonic acid ethyl ester, 5-aza-2'-deoxycytidine, Zeocin, methanesulfonic acid methyl ester, cis-diamminedichloro-platinum, mitomycin C, n-nitroso-n-ethylurea, gamma radiation, sodium azide, dimethylnitrosamine, bleomycin, 8-methoxypsoralen, cyclophosphamide, hydroxyurea, and diepoxybutane. The inventors also have provided chemical and physical treatments that effect chromatin structure including, but not limited to, n-butyric acid, heat shock, trichostatin A, cold stress, magnetic field, aflatoxin B1, and actinomycin D. Finally, the inventors describe Chemical and physical agents that effect proteins of the recombination machinery including, but not limited to, 3-methoxybenzamide or caffeine.

Several of these agents and their effect on recombination have been studied in mammalian and yeast cell systems. Studies in mammalian cells of agents that cause DNA damage or modifications include ultraviolet light (Bhattacharyya *et al.*, 1990), 1-nitrosopyrene (Bhattacharyya *et al.*, 1990), psoralen (Reardon *et al.*, 1991) and hydroxymethyl-trimethylpsoralen (HMT) (Vos and Hanawalt, 1989), cis-diamminedicloro-platinum or cisplatin (Hanneman *et al.*, 1997), cyclophosphamide (Schimenti *et al.*, 1997; Sykes *et al.*, 1998), bleomycin (Nakayama *et al.*, 1998), and sodium azide (Gonzalez-Cesar and Ramos-Morales, 1997). Studies of agents that affect chromatin structure in mammalian cells include sodium butyrate (Abramian *et al.*, 1994; Glebov *et al.*, 1994) and magnetic fields (Koana *et al.*, 1997). Studies of agents that affect the recombination machinery in mammalian cells include 3-methoxybenzamide (Waldeman *et al.*, 1996; Waldeman and Waldeman, 1991). Similar studies have been done in yeast including DNA modifying or damaging agents such as hydroxy urea (Galli and Schiestl, 1996) and psoralen (Dardalhon *et al.*, 1998; Saffran *et al.*, 1992) as well as chromatin altering agents, including aflatoxin (Kaplanski *et al.*, 1998) and actinomycin D (Nestmann *et al.*, 1981). In general, all of the above studies in both mammalian and yeast systems looked at mitotic recombination at a single locus. Often times this locus was an experimental transgene and not at all representative of genomic recombination activity as a whole.

The effects of a number of physical and chemical agents have also been examined in plant systems. Again, these can be classified into agents that cause DNA damage or modifications, including diepoxybutane (Sinha and Helagson, 1969), dimethylnitrosamine (Preidel and Schimmer, 1986), radiation (Vig, 1975; Tovar and Lichtenstein, 1992; Lebel *et al.*, 1993), mitomycin C (Vig, 1975; Lebel *et al.*, 1993), and UV light (Puchta *et al.*, 1995); agents that affect chromatin structure including actinomycin D (Vig, 1973 and 1975; Sinha and Helgason, 1969), colchicine (Vig, 1975), and heat shock (Maguire, 1968; Lebel *et al.*, 1993); and agents that affect the recombination machinery including caffeine (Vig, 1973 and 1975; Harrison and Carpenter, 1977) and 3 methoxybenzamide (Puchta *et al.*, 1995). Only three of the references looked at effects of the agents on meiotic recombination (Maguire, 1968;

Sinha and Helgason, 1969; Preidel and Schimmer, 1986), while all of the others were only concerned with mitotic recombination. Again, only a single locus was examined, which was oftentimes an experimental transgene, and not at all indicative of what was occurring in the genome as a whole.

There exists a clear need for chemical and physical treatments that will stimulate meiotic and mitotic recombination in plants. Stimulation of recombination would be beneficial for a number of applications including traditional plant breeding to form hybrids, self-pollinating plants in order to eliminate undesirable traits, introgression of a single trait from one plant line into another, generating novel mutations with novel desired phenotypes, genetic mapping experiments, and generation of transgenic plants by transformation with exogenous genetic material. Thus treatments that stimulate homologous as well as non-homologous recombination would be beneficial. Present methods used in all of these applications are dependent on, and therefore limited by, the rates of recombination in plants.

SUMMARY OF THE INVENTION

The present invention describes a method for increasing meiotic recombination in a plant by the following techniques. Plant strains 1 and 2 are crossed to generate an F1 hybrid. A chemical or physical treatment that results in DNA modification or damage is then applied to the F1 hybrid. This F1 hybrid is then used as a pollen donor or acceptor for pollination to generate seed, which are then propagated and the resulting F2 progeny identified or screened through for novel genetic recombinations (Figure 1). In specific embodiments, the treated F1 hybrid is self-pollinated. In other embodiments, the treated F1 hybrid is backcrossed a number of times resulting in introgression of a desired trait between strain 1 and strain 2. During each stage of backcrossing the crossed strains can be treated, thus further enhancing the desired recombination events and decreasing the number of backcross generations necessary. This method further comprises mapping of plant genes through increasing non-parental combinations of markers in the F2 progeny. Also

contemplated are methods for increasing meiotic recombination in a plant when strain 1 and strain 2 are of different species.

In specific embodiments, the plant is a gymnosperm, a crop, or an angiosperm. The angiosperm may be a monocot, and may be selected from a group consisting of maize, rice, wheat, barley, sorghum, oat, or sugarcane. Alternatively, the angiosperm is a dicot, and may be selected from the group consisting of cotton, tobacco, tomato, soybean, sunflower, oil seed rape (canola), alfalfa, pea, carrot, potato, strawberry, onion, broccoli, *Arabidopsis*, pepper, or citrus. In preferred embodiments, the plant is a member of the *Brassica* genus and is selected from the group consisting of cabbage, cauliflower, broccoli, Brussel sprout, kale, collard green, turnip, rutabaga, rapeseed or mustard. In other embodiments, the plant is *Arabidopsis thaliana.*

The chemical or physical treatment that results in DNA modification or damage is selected from a group consisting of U.V. exposure, methanesulfonic acid ethyl ester, 5-aza-2'-deoxycytidine, Zeocin, methanesulfonic acid methyl ester, cis-diamminedichloro-platinum, mitomycin C, n-nitroso-n-ethylurea, gamma radiation, sodium azide, dimethylnitrosamine, bleomycin, 8-methoxypsoralen, cyclophosphamide, hydroxyurea, or diepoxybutane. In addition, combinations of treatments are specifically contemplated.

The present invention describes a method for increasing meiotic recombination in a plant by the following techniques. Plant strains 1 and 2 are crossed to generate an F1 hybrid. Chemical or physical treatments that result in altering chromatin structure are then applied to the F1 hybrid. This F1 hybrid is then used as a pollen donor or acceptor in crosses to generate seed, which are then propagated and the resulting F2 progeny identified or screened for novel genetic recombinations. In specific embodiments, the treated F1 hybrid is self-pollinated. In other embodiments, the treated F1 hybrid is backcrossed a number of times resulting in introgression of a desired trait between strain 1 and strain 2. During each stage of backcrossing the crossed strains cab be treated, thus enhancing the desired recombination events and

decreasing the number of backcross generations necessary. This method further comprises mapping of plant genes through increasing non-parental combinations of markers in the F2 progeny. Also contemplated are methods for increasing meiotic recombination in a plant when strain 1 and strain 2 are of different species.

The chemical or physical treatment that results in altering chromatin structure is selected from a group consisting of n-butyric acid, heat shock, trichostatin A, cold stress, magnetic field, aflatoxin B1, or actinomycin D. In addition, combinations of treatments are specifically contemplated.

In specific embodiments where chemical or physical treatments that result in altering chromatin structure are used, the plant is a gymnosperm, a crop, or an angiosperm. The angiosperm may be a monocot, and may be selected from a group consisting of maize, rice, wheat, barley, sorghum, oat, or sugarcane. Alternatively, the angiosperm is a dicot, and may be selected from the group consisting of cotton, tobacco, tomato, soybean, sunflower, oil seed rape (canola), alfalfa, potato, strawberry, onion, broccoli, *Arabidopsis*, pepper, or citrus. In preferred embodiments, the plant is a member of the *Brassica* genus and is selected from the group consisting of cabbage, cauliflower, broccoli, Brussel sprout, kale, collard green, turnip, rutabaga, rapeseed or mustard. In other embodiments, the plant is *Arabidopsis thaliana.*

The present invention describes a method for increasing meiotic recombination in a plant by the following techniques. Plant strains 1 and 2 are crossed to generate a F1 hybrid. Chemical or physical treatments that result in changes to the specificity or activity of the recombination machinery are then applied to the F1 hybrid. This F1 hybrid is then used to pollinate or be pollinated to generate seed, which are then propagated and the resulting F2 progeny identified or screened through for novel genetic recombinations. In specific embodiments, the treated F1 hybrid is self-pollinated. In other embodiments, the treated F1 hybrid is backcrossed a number of times resulting in introgression of a desired trait between strain 1 and strain 2. During each stage of backcrossing the crossed strains cab be treated, thus enhancing

the desired recombination events and decreasing the number of backcross generations necessary. This method further comprises mapping of plant genes through increasing non-parental combinations of markers in the F2 progeny. Also contemplated are methods for increasing meiotic recombination in a plant when strain 1 and strain 2 are of different species. The chemical or physical treatment that results in altering the recombination machinery is 3-methoxybenzamide or caffeine, or a combination of the two.

In specific embodiments where chemical or physical treatments that result in altering recombination machinery are used, the plant is a gymnosperm, a crop, or an angiosperm. The angiosperm may be a monocot, and may be selected from a group consisting of maize, rice, wheat, barley, sorghum, oat, or sugarcane. Alternatively, the angiosperm is a dicot, and may be selected from the group consisting of cotton, tobacco, tomato, soybean, sunflower, oil seed rape (canola), alfalfa, potato, strawberry, onion, broccoli, *Arabidopsis*, pepper, or citrus. In preferred embodiments, the plant is a member of the *Brassica* genus and is selected from the group consisting of cabbage, cauliflower, broccoli, Brussel sprout, kale, collard green, turnip, rutabaga, rapeseed or mustard. In other embodiments, the plant is *Arabidopsis thaliana.*

Also described is a method of increasing mitotic recombination in a plant by causing DNA modification or damage, altering chromatin structure, or altering recombination machinery of said plant by treating with a chemical or physical agent followed by propagating of said plant vegetatively, or by producing seed, either through self or cross pollination.

Methods for increasing transformation of a plant with a genetic element by causing DNA modification or damage, altering chromatin structure, or altering recombination machinery of the plant by treating with a chemical or physical agent followed by propagating the plant are also disclosed. Specifically, the transformation and treating with a chemical or physical agent of the plant may be simultaneous, the transformation may be prior to treating with a chemical or physical agent of the plant,

or the transformation follows treating with a chemical or physical agent of the plant. The transformation may proceed via non-homologous or homologous recombination. In preferred embodiments, the transformation is via *Agrobacterium*, particle bombardment, liposome fusion, electroporation, microinjection, or is polyethylene glycol mediated.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1: Parent 1 (genotype AAbb) and Parent 2 (genotype aaBB) are crossed to generate an F1 hybrid with the genotype AaBb. Markers A and B are separated by 2 cM therefore one would expect 2% of all meiosis in the F1 would have a recombination event between the two markers producing recombinant gametes with the haploid genotype ab or AB. If the F1 hybrid was treated in such a way as to stimulate recombination by 10 fold, 20% of all meiosis in the F1 would be expected to have a recombination event between the two markers producing recombinant gametes with the haploid genotype ab or AB.

DETAILED DESCRIPTION OF THE INVENTION

The present invention describes a number of physical or chemical treatments that increase the frequency of recombination in a plant. These treatments act to either cause DNA damage or modifications, affect chromatin structure, or affect the recombination machinery in the cell. Increases in the frequency of recombination are useful for improving a number of manipulations with plants including traditional plant breeding to form hybrids, self-pollinating plants in order to eliminate undesirable traits, introgression of a single trait from one plant line into another, generating novel

mutations with novel desired phenotypes, genetic mapping experiments, and generation of transgenic plants by transformation with exogenous genetic material. Both homologous and non-homologous recombination frequencies are increased, as well as an increased frequency of recombination during meiosis and mitosis.

I. Recombination

DNA sequences are rearranged by a process called recombination. Recombination serves many purposes in biologic systems. These include the maintenance of genetic diversity, DNA repair, the regulation of expression of certain genes, and programmed genetic rearrangements during development are a few of the naturally occurring roles of genetic recombination events. Recombination also is utilized in many experimental and technology related roles where there is a desire to manipulate the genomes of various organisms, including genetic transformation, genetic crosses and breeding for desired traits, as well as mapping, identifying and isolating novel genetic traits.

Recombination can be grouped into four general categories. The first is homologous recombination which involves genetic exchanges between any two DNA segments that share an extended region of homology. The actual sequences of DNA are not relevant as long as the sequences in the two DNA molecules are similar. The second is site-specific recombination where recombination occurs at defined DNA sequences. The third is random recombination, generally involving the integration of DNA from one source into a chromosome at an essentially random site. The fourth category is DNA transpositions, where a short region of DNA has the ability to move from one location in the genome to another. The present invention describes methodologies and applications that potentially involve all four of these general areas of recombination.

There are various mechanisms involving recombination that lead to the production of new arrangements of genes including assortment and segregation, crossing over, gene conversion, transformation, conjugation, transduction, or mixed

infection of viruses. Crossing over is the reciprocal exchange of segments at corresponding positions along pairs of homologous chromosomes by symmetrical breakage and crosswise rejoining. Other crossing-over events involve an exchange of segments between the sister chromatids of a chromosome, either between the sister chromatids of a meiotic tetrad or between the sister chromatids of a duplicated somatic chromosome. Aberrant crossing over can occur in regions of limited or no homology. Gene conversion results from a recombination and repair event that converts one allele to another which leads to the production of non-reciprocal recombinant strands. For example, the meiotic products of an Aa individual following chromosome replication would be expected to be AAaa. Gene conversion results in novel arrangements of the alleles (such as Aaaa or AAAa rather than AAaa) following replication and recombination. Transformation is the unidirectional transfer and incorporation of DNA by prokaryotic or eukaryotic cells and the subsequent recombination of part or all of that DNA into the cell's genome. Transduction is the transfer of foreign DNA by phages or viruses from the infected cell in which the DNA originates to another cell.

A. Meiotic Recombination

Many recombination events occur during meiosis. Meiosis is a special method of cell division, occurring during the maturation of the germ-line cells, by means of which each daughter nucleus receives half the number of chromosomes characteristic of the somatic cells of the species. In general, meiosis begins with the replication of the DNA in the germ-line cell so that each DNA molecule is present in four copies. The cell then undergoes two meiotic cell divisions that reduce the DNA content to the haploid level in the four resulting daughter cells.

Following the initial DNA replication, the resulting DNA copies remain associated with each other at their centromeres and are referred to as "sister" chromatids. Each set of four homologous DNA molecules is arranged as two pairs of chromatids. Homologous recombination occurs at this stage between the closely aligned homologous chromatids. This process involves breakage and rejoining of

DNA resulting in genetic exchanges called crossovers. Crossing over temporarily links all four homologous chromatids at points called chiasmata. To a first approximation, crossing over can occur with equal probability at almost any point along the length of two homologous chromosomes. The frequency of recombination on a given chromosome between two points is directly proportional to the distance between the two points. Genetic mapping of the relative positions of genes makes use of the recombination frequency between any two genes. The frequency of crossing over between chromosomes is lower in some regions of the chromosome, in particular the regions around the centromere. The consequences of recombination during meiosis are at least threefold. The first is an increase in genetic diversity, the second is providing a physical link between chromosomes allowing orderly segregation of the chromosomes to the daughter cells, and the third is repair of several types of DNA damage.

The present invention seeks to increase the rate of meiotic recombination by various physical and chemical treatment of organisms, and in particular plants. Increased meiotic recombination would be beneficial in many aspects of plant breeding, including introgression of genetic traits, producing novel hybrids, especially between two plant species, and mapping of plant genes.

B. Mitotic Recombination

Cell mitosis is a multi-step process that includes DNA replication and cell division (Alberts *et al.*, 1989; Stryer, 1988). Mitosis is characterized by the establishment of mitotic spindles and the intracellular movement and segregation of chromosomes and organelles.

Mitosis is a method of cell division in which the two daughter nuclei normally receive identical complements of the number of chromosomes of the somatic cells of the species. Mitosis occurs following the duplication of each chromosome and then pairing of the chromosomes into sister chromatids. The two chromatids then segregate to opposite poles of the cell. Finally, the cell splits in two by a process

called cytokinesis, resulting in two daughter cells with a complete diploid complement of chromosomes. Like meiosis, recombination can occur between sister chromatids and if those sisters have a different DNA composition can result in novel combinations of genetic elements in the two daughter cells. Mitosis is the process where multicellular organisms replicate their somatic cells. Mitosis also is the process of cell division under *in vitro* conditions such as tissue culture.

Increasing mitotic recombination frequencies would again be beneficial to many aspects of plant breeding and other manipulations. For example, any manipulation that requires chromosome breakage and translocation, transformation or integration of DNA at a homologous or non-homologous site will benefit from increased frequencies of recombination. Increased rates of homologous transformation would be extremely beneficial, especially in plant systems.

C. Homologous and Non-homologous Recombination

Non-homologous recombination occurs between strands of DNA where there is no appreciable or limited sequence homology. Examples of non-homologous recombination include insertions, deletions and other rearrangements. Non-homologous recombination can be between host DNA and host DNA, between host DNA and foreign DNA, or between foreign DNA already in a host and new foreign DNA. Non-homologous DNA integration is generally random, although preferences or hot-spots for integration are often times observed. It is specifically contemplated by the inventors that one could employ techniques or treatments to increase the frequency of non-homologous recombination. Non-homologous recombination, such as insertion of foreign DNA into a host DNA during transfection, occurs at low frequencies. Many techniques are available for delivering DNA, or other genetic material such as RNA, to a recipient cell and are outlined in the section below on transformation. What is frequently rate-limiting in this transformation event is integration of the genetic materials into the host DNA resulting in stable transformation.

Homologous recombination relies on recombination machinery that can recognize complementary nucleic acids. In this instance, base pairing can serve to facilitate the interaction of two separate nucleic acid molecules so that strand breakage and repair can take place. In other words, the "homologous" aspect of the method relies on sequence homology to bring complementary sequences into close proximity, while the "recombination" aspect provides for one complementary sequence to replace the other by virtue of the breaking of certain bonds and the formation of others.

It also is specifically contemplated by the inventors that one could employ techniques or treatments to increase the frequency of homologous recombination for gene or target specific replacement or "knock out" of genes. For this situation, a target gene is selected within the host cell. Sequences homologous to the target gene are then included in a genetic construct, along with some mutation that will render the target gene inactive (stop codon, interruption, *etc.*). The homologous sequences to either side of the inactivating mutation are said to "flank" the mutation. Flanking, in this context, simply means that target homologous sequences are located both upstream (5') and downstream (3') of the mutation. These sequences should correspond to some sequences upstream and downstream of the target gene. The construct is then introduced into the cell, thus permitting homologous recombination between the cellular sequences and the construct.

Integration or excision of transgenes or parts of transgenes can be achieved in plants by means of homologous recombination (see, for example, U.S. Patent No. 5,527,695, specifically incorporated herein by reference in its entirety). Homologous recombination is a reaction between any pair of DNA sequences having a similar sequence of nucleotides, where the two sequences interact (recombine) to form a new recombinant DNA species. The frequency of homologous recombination increases as the length of the shared nucleotide DNA sequences increases, and is higher with linearized plasmid molecules than with circularized plasmid molecules. Homologous recombination can occur between two DNA sequences that are less than identical, but

the recombination frequency declines as the divergence between the two sequences increases.

Introduced DNA sequences can be targeted via homologous recombination by linking a DNA molecule of interest to sequences sharing homology with endogenous sequences of the host cell. Once the DNA enters the cell, the two homologous sequences can interact to insert the introduced DNA at the site where the homologous genomic DNA sequences were located. Therefore, the choice of homologous sequences contained on the introduced DNA, will determine the site where the introduced DNA is integrated via homologous recombination. For example, if the DNA sequence of interest is linked to DNA sequences sharing homology to a single copy gene of a host plant cell, the DNA sequence of interest will be inserted via homologous recombination at only that single specific site. However, if the DNA sequence of interest is linked to DNA sequences sharing homology to a multicopy gene of the host eukaryotic cell, then the DNA sequence of interest can be inserted via homologous recombination at each of the specific sites where a copy of the gene is located.

DNA can be inserted into the host genome by a homologous recombination reaction involving either a single reciprocal recombination (resulting in the insertion of the entire length of the introduced DNA) or through a double reciprocal recombination (resulting in the insertion of only the DNA located between the two recombination events). For example if one wishes to insert a foreign gene into the genomic site where a selected gene is located, the introduced DNA should contain sequences homologous to the selected gene. A single homologous recombination event would then result in the entire introduced DNA sequence being inserted into the selected gene. Alternatively, a double recombination event can be achieved by flanking each end of the DNA sequence of interest (the sequence intended to be inserted into the genome) with DNA sequences homologous to the selected gene. A homologous recombination event involving each of the homologous flanking regions will result in the insertion of the foreign DNA. Thus only those DNA sequences

located between the two regions sharing genomic homology become integrated into the genome.

As a practical matter, the genetic construct will normally act as far more than a vehicle to interrupt the gene. For example, it is important to be able to select for recombinants and, therefore, it is common to include within the construct a selectable marker gene. This gene permits selection of cells that have integrated the construct into their genomic DNA by, for example, conferring resistance to various biostatic and biocidal drugs. In addition, a heterologous gene that is to be expressed in the cell also may advantageously be included within the construct. Thus, using this kind of construct, it is possible, in a single recombination event, to (i) "knock out" an endogenous gene, (ii) provide a selectable marker for identifying such an event and (iii) introduce another allele of the same gene or a heterologous gene for expression.

Another refinement of the homologous recombination approach involves the use of a "negative" selectable marker. This marker, unlike the selectable marker, causes death of cells which express the marker. Thus, it is used to identify undesirable recombination events. When seeking to select homologous recombinants using a selectable marker, it can be difficult in the initial screening step to identify proper homologous recombinants from recombinants generated from random, non-sequence specific events. These recombinants also may contain the selectable marker gene and may express the heterologous protein of interest, but will, in all likelihood, not have the desired "knock out" phenotype. By attaching a negative selectable marker to the construct, but outside of the flanking regions, one can select against many random recombination events that will incorporate the negative selectable marker. Homologous recombination should not introduce the negative selectable marker, as it is outside of the flanking sequences.

Although introduced sequences can be targeted for insertion into a specific genomic site via homologous recombination, in higher eukaryotes homologous recombination is a relatively rare event compared to random insertion events which

occur at a frequency as much as 10^5 or 10^6 greater than homologous insertion events. In plant cells, foreign DNA molecules find homologous sequences in the cell's genome and recombine at a frequency of approximately 0.5×10^{-4} to 4.2×10^{-4}. Only a few cases of homologous recombination events in plants have been described.

The inventors also specifically contemplate that one could employ techniques or treatments to increase the frequency of recombination in site-specific recombinase systems, including, but not limited to, Cre-lox and FLP/FRT systems. In general, a site specific recombinase system consists of three elements: two pairs of DNA sequence (the site-specific recombination sequences) and a specific enzyme (the site-specific recombinase). The site-specific recombinase will catalyze a recombination reaction only between two site-specific recombination sequences.

An advantage of site-specific integration or excision is that it can be used to overcome problems associated with conventional transformation techniques, in which transgenes typically randomly integrate into a host genome and in multiple copies. This random insertion of introduced DNA into the genome of host cells can be lethal if the foreign DNA inserts into an essential gene. In addition, the expression of a transgene may be influenced by "position effects" caused by the surrounding genomic DNA. Further, because of difficulties associated with transformation of multiple transgene copies, including gene silencing, recombination and unpredictable inheritance, it is typically desirable to control the copy number of the inserted DNA, often only desiring the insertion of a single copy of the DNA sequence.

A number of different site-specific recombinase systems could be employed in accordance with the instant invention, including, but not limited to, the Cre-lox system of bacteriophage P1 (U.S. Patent No. 5,658,772, specifically incorporated herein by reference in its entirety), the FLP/FRT system of yeast (Golic and Lindquist, 1989), the Gin recombinase of phage Mu (Maeser *et al.*, 1991), the Pin recombinase of *E. coli* (Enomoto *et al.*, 1983), and the R/RS system of the pSR1 plasmid (Araki *et al.*, 1992). The bacteriophage P1 Cre/lox and the yeast FLP/FRT systems constitute

two particularly useful systems for site specific integration or excision of transgenes. In these systems a recombinase (Cre or FLP) will interact specifically with its respective site -specific recombination sequence (lox or FRT respectively) to invert or excise the intervening sequences. The sequence for each of these two systems is relatively short (34 bp for lox and 47 bp for FRT) and therefore, convenient for use with transformation vectors.

The FLP/FRT and Cre-lox recombinase systems have been demonstrated to function efficiently in plant cells, but this efficiency could potentially be increased by application of the techniques described herein. Studies on the performance of the FLP/FRT system in both maize and rice protoplasts indicate that FRT site structure, and amount of the FLP protein present, affects excision activity. In general, short incomplete FRT sites leads to higher accumulation of excision products than the complete full-length FRT sites. The systems can catalyze both intra- and intermolecular reactions in maize protoplasts, indicating its utility for DNA excision as well as integration reactions. The recombination reaction is reversible and this reversibility can compromise the efficiency of the reaction in each direction. Altering the structure of the site-specific recombination sequences is one approach to remedying this situation. The site-specific recombination sequence can be mutated in a manner that the product of the recombination reaction is no longer recognized as a substrate for the reverse reaction, thereby stabilizing the integration or excision event.

In the Cre-lox system, discovered in bacteriophage P1, recombination between loxP sites occurs in the presence of the Cre recombinase (see, *e.g.*, U.S. Patent No. 5,658,772, specifically incorporated herein by reference in its entirety). This system has been utilized to excise a gene located between two lox sites which had been introduced into a yeast genome (Sauer, 1987). Cre was expressed from an inducible yeast GAL1 promoter and this Cre gene was located on an autonomously replicating yeast vector. Successful use of the Cre-lox system in plants was described, for example, by Osborne (1995).

Since the lox site is an asymmetrical nucleotide sequence, lox sites on the same DNA molecule can have the same or opposite orientation with respect to each other. Recombination between lox sites in the same orientation results in a deletion of the DNA Segment located between the two lox sites and a connection between the resulting ends of the original DNA molecule. The deleted DNA segment forms a circular molecule of DNA. The original DNA molecule and the resulting circular molecule each contain a single lox site. Recombination between lox sites in opposite orientations on the same DNA molecule result in an inversion of the nucleotide sequence of the DNA segment located between the two lox sites. In addition, reciprocal exchange of DNA segments proximate to lox sites located on two different DNA molecules can occur. All of these recombination events are catalyzed by the product of the Cre coding region. Similar strategies can be employed using the FLP/FRT system since FRT is also and asymmetrical nucleotide sequence.

II. DNA Modification or Damage

When a cell undergoes reproduction, its DNA molecules are replicated and precise copies are passed on to its descendants. The linear base sequence of a DNA molecule is maintained in the progeny during replication in the first instance by the complementary base pairings which allow each strand of the DNA duplex to serve as a template to align free nucleotides with its polymerized nucleotides. The complementary nucleotides so aligned are biochemically polymerized into a new DNA strand with a base sequence that is entirely complementary to that of the template strand.

Occasionally, incorrect bases are incorporated during replication, which, after further replication of the new strand, results in a double-stranded DNA offspring with a DNA strand containing a heritable sequence different from that of the parent DNA molecule. Such heritable changes are called genetic mutations, or more particularly in the present case, "single base pair" or "point" mutations. The consequences of a point mutation may range from negligible to lethal, depending on the location and effect of the sequence change in relation to the genetic information encoded by the DNA.

The bases A and G are of a class of compounds called purines, while T and C are pyrimidines. Whereas the normal base pairings in DNA (A with T, G with C) involve one purine and one pyrimidine, the most common single base mutations involve substitution of one purine or pyrimidine for the other (*e.g.*, A for G or C for T or vice versa), a type of mutation referred to as a "transition". Mutations in which a purine is substituted for a pyrimidine, or vice versa, are less frequently occurring and are called "transversions". Still less common are point mutations comprising the addition or loss of a small number (1, 2, 3 or more) of nucleotides arising in one strand of a DNA duplex at some stage of the replication process. Such mutations are called small "insertions" or "deletions", respectively, and also are known as "frameshift" mutations when they occur in sequences coding for proteins, due to their effects on translation of the genetic code. Mutations involving larger sequence rearrangement also occur and can be important in medical genetics, but their occurrences are relatively rare compared to the classes summarized above

Recombination is an important part of DNA repair necessitated by DNA modifications or damage when the necessary sequence information is not available from a strand of DNA paired with the damaged strand. Examples of such damage or modifications requiring recombination include double-strand breaks, double strand cross-links, lesions in single standed DNA, and base pair mismatches. These types of modifications or damage induce the cellular recombination machinery to help make the repairs. This is the mechanism whereby chemical and physical treatments that cause DNA modifications and damage increase the frequency of recombination in a cell. Examples of chemical and physical treatments that cause DNA modification and damage include, but are not limited to, U.V. exposure, methanesulfonic acid ethyl ester, 5-aza-2'-deoxycytidine, Zeocin, methanesulfonic acid methyl ester, cis-diamminedichloro-platinum, mitomycin C, n-nitroso-n-ethylurea, gamma radiation, sodium azide, dimethylnitrosamine, bleomycin, 8-methoxypsoralen, cyclophosphamide, hydroxyurea, and diepoxybutane.

III. Chromatin Structure

Chromatin is a DNA-protein complex essential for eukaryotic cell differentiation and function. Its structure is maintained and regulated in part by a family of basic proteins, named histones. Histones are small proteins with molecular masses ranging from 11 kDa to 22 kDa. They are remarkably conserved in sequence and structure across species indicating their critical role in gene evolution. Five types of histones, termed H1, H2A, H2B, H3, and H4, exist in a variety of forms due to post-translational modifications of certain side chains. A striking common feature of histones is their high content of positively charged side chains, about one in four residues is either lysine or arginine. When a histone-DNA complex forms, the positive charges of the histone side chains neutralize the negative charges of chromosomal DNA. Specifically, H2A, H2B, H3, and H4 interact with repeats of 200 base pairs of chromosomal DNA to form nucleosomes. Then, H1 interacts with H2A to group the nucleosomes into second order structure. Higher-order structure of chromosomes involves the interaction of histones and chromosomal DNA with a series of nonhistone proteins.

Each of the histones can exist in different forms due to post-translational modifications of the amino acid side chains. These modifications include methylation, ADP-ribosylation, phosphorylation, acetylation and ubiquitination. These modifications change the histones molecule's net electric charge, shape and other properties and hence have effects on the DNA they are interacting with. The DNA is wound around the histones in such a way as to tighten or loosen the supercoiling of the DNA helix. The extent of supercoiling plays a role in the transcriptional activity of the region of DNA as well as the DNA's availability for recombination. Accessory enzymes such as topoisomerases play a role in the extent of supercoiling. Regions of the genome that are very transcriptionally active tend to have a deficiency of histone H1, a greater tendency for acetylation of the remaining histones and an overall undermethylation of the DNA bases. In general, transcriptionally active chromatin is more open, with less structural barriers. These same regions of active chromatin are thought to be more accessible to recombination.

Factors that affect the overall chromatin structure, whether acting directly on histone modification, topoisomerase activity and DNA supercoiling, or through general increases in transcriptional activity will all likely influence the frequency of recombination. Examples of chemical and physical treatments that influence chromatin structure and can increase recombination frequency include, but are not limited to, n-butyric acid, heat shock, trichostatin A, cold stress, magnetic field, aflatoxin B1, and actinomycin D.

IV. Recombination Machinery

Enzymes have been isolated from both prokaryotes and eukaryotes that promote, or are involved, in one or more aspects of recombination. Much of the progress in identifying and understanding these enzymes has come from studies in *E. coli*. Important recombination enzymes are encoded by the *rec*A, B, C and D genes, and by the *ruv*C genes of *E. coli*. The enzymes encoded by these genes are involved in several steps of recombination, including unwinding the DNA helix, strand breakage, pairing of homologous DNA's, branch migration, and resolution of recombination intermediate structures. Analogs of these *E. coli* genes in other biologic systems, including yeast, mammals and plants, is ongoing. More details on the enzymes involved in recombination are given below in the section on recombinase proteins.

The present invention details chemical and physical treatments that can influence the recombination machinery and lead to an increased frequency of recombination. Examples of treatments that affect the recombination machinery and increase recombination frequency include, but are limited to, 3-methoxybenzamide and caffeine.

A. Recombinase Proteins

Recombinases are proteins that are actively engaged in promoting or modulating recombination (homologous or nonhomologous) and can act within cells

or in purified enzymatic reactions in vitro. When included with an exogenous targeting polynucleotide, recombinases provide a measurable increase in the recombination frequency and/or localization frequency between the targeting polynucleotide and an endogenous predetermined DNA sequence. In the present invention, recombinase refers to a family of RecA-like recombination proteins, or any other recombination protein, having essentially all or most of the same functions, particularly: (i) the protein's ability to properly position targeting polynucleotides on their homologous or nonhomologous targets and (ii) the ability of recombinase-polynucleotide complexes to efficiently find and bind to DNA sequences targeted for recombination.

The best characterized recombinase protein is RecA from *E. coli*, in addition to the wild-type protein a number of mutant recA-like proteins have been identified (*e.g.*, recA803). Further, many organisms have recA-like strand-transfer proteins (*e.g.*, Fugisawa *et al.*, 1985; Hsieh *et al.*, 1986; Hsieh *et al.*, 1989; Fishel *et al.*, 1988; Cassuto *et al.*, 1987; Ganea *et al.*, 1987; Moore *et al.*, 1990; Keene *et al.*, 1984; Kimiec, 1984; Kolodner *et al.*, 1987; Sugino *et al.*, 1985; Halbrook *et al.*, 1989; Eisen *et al.*, 1988; McCarthy *et al.*, 1988; Lowenhaupt *et al.*, 1989). Examples of such recombinase proteins include, for example but not limitation: recA, recA803 and other recA mutants (Roca, 1990), sep1 (Kolodner *et al.*, 1987; Tishkoff *et al.*,1991), RuvC (Dunderdale *et al.*, 1991), DST2, KEM1, XRN1 (Dykstra *et al.*, 1991), STPα/DST1 (Clark *et al.*, 1991), HPP-1 (Moore *et al.*, 1991), uvsX, and DMC1 (Kimyuk, 1997). RecA may be purified from *E. coli* strains, such as *E. coli* strains JC12772 and JC15369. These strains contain the recA coding sequences on a "runaway" replicating plasmid vector present at a high copy numbers per cell. The recA803 protein is a high-activity mutant of wildtype recA. The art teaches several examples of recombinase proteins from Drosophila, plant, human, and non-human mammalian cells, including proteins with biological properties similar to recA (*i.e.*, recA-like recombinases).

Recombinase protein(s) may be exogenously administered to a eukaryotic cell simultaneously or contemporaneously (*i.e.*, within about a few hours) with the

targeting polynucleotide(s). Such administration typically is performed by microinjection, although electroporation, lipofection, and other methods known in the art may also be used. Alternatively, recombinase proteins may be produced *in vivo* from a heterologous expression cassette in a transfectant cell or transgenic cell, such as a transgenic totipotent embryonal stem cell (*e.g.*, a murine ES cell such as AB-1) used to generate a transgenic non-human animal line or a pluripotent hematopoietic stem cell for reconstituting all or part of the hematopoietic stem cell population of an individual. Conveniently, a heterologous expression cassette includes a modulatable promoter, such as an ecdysone-inducible promoter-enhancer combination, an estrogen-induced promoter-enhancer combination, a CMV promoter-enhancer, an insulin gene promoter, or other cell-type specific, developmental stage-specific, hormone-inducible, or other modulatable promoter construct so that expression of at least one species of recombinase protein from the cassette can by modulated for transiently producing recombinase(s) *in vivo* simultaneous or contemporaneous with introduction of a targeting polynucleotide into the cell. When a hormone-inducible promoter-enhancer combination is used, the cell must have the required hormone receptor present, either naturally or as a consequence of expression a co-transfected expression vector encoding such receptor.

Specific examples of recombinase transgene effects on recombination in plants include RecA (Reiss *et al.*, 1996), HO endonuclease (Chiurazzi *et al.*, 1996), and Sce I endonuclease (U.S. Patent 5,830,729; Puchta *et al.*, 1996).

V. Chemical and Physical Treatments

Several chemical and physical treatments that effect the recombination frequency in cells are described in this application, and are grouped into three categories: treatments that affect recombination through DNA modification and damage; treatments that affect recombination through effects on chromatin structure; and treatments that effect recombination through effects on the recombination machinery. The following section details examples of treatments in each category.

A. Treatments Resulting in DNA Modification and Damage

i) U.V. exposure

That portion of the electromagnetic spectrum immediately below the visible range and extending into the x-ray frequencies. The longer wavelengths (near-UV or biotic or vital rays) are necessary for the endogenous synthesis of vitamin D and are also called antirachitic rays; the shorter, ionizing wavelengths (far-UV or abiotic or extravital rays) are viricidal, bactericidal, mutagenic, and carcinogenic and are used as disinfectants. Exposure of DNA to U.V. irradiation at ionizing wavelengths produces thymine dimers and other aberrant bonds in which the normal chemical structure of DNA is altered. These DNA lesions cause distortion in the DNA helix beyond which replication cannot proceed and must be repaired or the cell dies.

ii) Gamma radiation

Very powerful and penetrating, high-energy electromagnetic radiation of shorter wavelength than that of x-rays. They are emitted by a decaying atomic nucleus, usually between 0.01 and 10 MeV. They are also called nuclear x-rays.

iii) Methanesulfonic acid ethyl ester

Methanesulfonic acid, ethyl ester. An antineoplastic agent with alkylating properties. It also acts as a mutagen by damaging DNA and is used experimentally for that effect.

iv) 5-aza-2'-deoxycytidine

Deoxy version of azacytidine, having antibiotic and antineoplastic activity. A pyrimidine analogue that inhibits DNA methyltransferase, impairing DNA methylation. It is also an antimetabolite of deoxycytidine, incorporated primarily into DNA. This compound could also be used as a modifier of chromatin structure.

v) Zeocin

Zeocin ™ is a formulation of phleomycin D1, a basic, water-soluble, copper-chelated glycopeptide isolated from *Streptomyces verticillus* (Invitrogen Inc.,

Carlsbad, CA). The copper-chelated form is inactive. When the antibiotic enters the cell, the copper cation is reduced from Cu^{2+} to Cu^{1+} and removed by sulfhydryl compounds in the cell. Upon removal of the copper, Zeocin ™ is activated and will bind DNA and cleave it, causing cell death. It shows strong toxicity against bacteria, fungi, plants and mammalian cells.

vi) Methanesulfonic acid methyl ester

Methanesulfonic acid, methyl ester. An alkylating agent in cancer therapy that may also act as a mutagen by interfering with and causing damage to DNA.

vii) Cis-diamminedichloro-platinum

cis-Diamminedichloroplatinum (II) (cis-DDP or cisplatin) is a clinically important antitumor drug used mainly to combat ovarian and testicular malignancies (Loehrer and Einhorn, 1984). The major cellular target for cis-DDP is generally accepted to be DNA, although it is not yet certain whether antitumor efficacy is a consequence of impaired replication or transcription (Sorenson and Eastman, 1988). Covalent coordination of the hydrolysis products of cis-DDP to the bases in DNA can lead to inhibition of DNA synthesis in vitro and in vivo and cause mutagenesis (Lee and Martin, 1976; Lim and Martini, 1984; Pinto and Lippard, 1985; Harder and Rosenberg, 1970; Howle and Gale, 1970; Burnouf *et al.*, 1987).

viii) Mitomycin C

Mitomycin C is an antibiotic which is produced by fermentation and is presently on sale under Food and Drug Administration approval in the therapy of disseminated adenocarcinoma of the stomach or pancreas in proven combinations with other approved chemotherapeutic agents and as palliative treatment when other modalities have failed (Mutamycin R Bristol Laboratories, Syracuse, N.Y. 13201, Physicians' Desk Reference 35th Edition, 1981, pp. 717 and 718). Mitomycin C and its production by fermentation is the subject of U.S. Pat. No. 3,660,578 patented May 2, 1972. Mitomycin C reacts covalently with DNA forming crosslinks between the complementary strands of DNA.

ix) N-nitroso-n-ethylurea

A nitrosamine derivative (also known as N-Ethyl-N-nitrosoethanamine) with alkylating, carcinogenic, and mutagenic properties. It is a member of a highly reactive group of chemicals that introduce alkyl radicals into biologically active molecules and thereby prevent their proper functioning. Many are used as antineoplastic agents, but most are very toxic, with carcinogenic, mutagenic, teratogenic, and immunosuppressant actions.

x) Sodium azide

Sodium azide is a carcinogen, with the azide moiety (N_3^-) being highly reactive with DNA. It is also a cytochrome oxidase inhibitor which is a nitridizing agent and an inhibitor of terminal oxidation.

xi) Dimethylnitrosamine

Another nitrosamine derivative (also called N-Nitrosodimethylamine) with alkylating, carcinogenic, and mutagenic properties. It causes serious liver damage and is a hepatocarcinogen in rodents.

xii) Bleomycin

The antineoplastic agent bleomycin (BLM) refers to a group of peptides with antitumor activity widely used in the treatment of various cancers, such as squamous cell carcinoma, testicular carcinoma and Hodgkin's disease (Lazo *et al.*, 1989a; Carter, 1985). The cellular determinants responsible for regulating the toxicity of BLM remain unclear. However, the primary target for the therapeutic action of the BLM class of compounds is thought to be nuclear DNA (Umezawa, 1979; Lazo *et al.*, 1989b). It is believed, for example, that bleomycin causes strand scission and fragmentation of DNA and may, to a lesser extent, inhibit synthesis of RNA.

xiii) 8-methoxypsoralen

Psoralens are the linear isomers of the furocoumarin family and they occur naturally in certain fruits and seeds, *e.g.*, *Ammi majus* and *Psoralea corylifolia.*

Extracts of these fruits and seeds have been used since ancient times as dermal sensitizing agents in the treatment of vitiligo. Topical application of psoralen extracts, followed by irradiation with light, results in a stimulation of melanin production, thus producing a dermal "tanning" effect. Psoralens have been investigated with respect to their ability to form covalent bonds with nucleic acids. Because of their planar structure, psoralens can intercalate between the base pairs in the double helix molecular structure of nucleic acids. Upon irradiation with light of the proper wavelength, the psoralens may form covalent bonds with pyrimidine nucleotides that occur as integral entities of nucleic acid strands. Achieving covalently bonded psoralen bridges or crosslinks between the nucleic acid strands of the double helix presents another tool for use in studying, in vivo, secondary structures of nucleic acids.

xiv) Cyclophosphamide

Cyclophosphamide is one of the most significant antineoplastic drugs of today. Cyclophosphamide is disclosed and claimed in U.S. Patent No. 3,018,302 granted Jan. 23, 1962 to H. Arnold *et al.*, and is denoted chemically as 2-[bis(2-chloroethyl)amino]tetrahydro-2H-1,3,2-oxazaphosphorine -2-oxide monohydrate. Cyclophosphamid is metabolized into alkylating agents which cause DNA damage.

xv) Hydroxyurea

Hydroxyurea, a ribonucleotide reductase inhibitor, is a simple chemical compound ($CH_4N_2O_2$, MW 76.05) that was initially synthesized in the late 1800's (Calabresi and Chabner, 1990). It was later found to produce leukopenia in laboratory animals and subsequently was tested as an antineoplastic agent (Rosenthal *et al.*, 1928). At present, the primary clinical role of hydroxyurea is in the treatment of myeloproliferative disorders. It now is considered the preferred initial therapy for chronic myelogenous leukemia (Donehower, 1990). Hydroxyurea is thought to cause DNA strand breaks. It has also been observed to inhibit DNA repair and therefore can also be considered to effect the recombination machinery.

xvi) **Diepoxybutane**

1,2:3,4-diepoxybutane is a highly reactive crossslinking agent due to the two epoxide groups. It is a carcinogen due to its ability to react and form adducts with DNA.

B. Treatments Effecting Chromatin Structure

i) **N-butyric acid**

A four carbon acid, $CH_3CH_2CH_2COOH$, with an unpleasant odor that occurs in butter and animal fat as the glycerol ester. Butyric acid has a general effect on transcription in mammalian cells by relaxing the overall chromatin structure.

ii) **Heat shock**

A constellation of responses that occur when an organism is exposed to excessive heat and other environmental stresses. Responses include synthesis of some proteins, repression of other proteins, and expression of new proteins. (From Segen, Dictionary of Modern Medicine, 1992). Heat shock may also disrupt chromatin structure, opening up accessibility to DNA.

iii) **Trichostatin A**

A member of a group of trichostatin compounds, which include trichostatin A (TSA), trichostatin B, and trichostatin C, as well as derivatives thereof. These compounds are able to induce high acetylation of histones. TSA is a substance which was first found as an antibiotic against a range of fungi, and at present, is used as a cell growth inhibitor specific to the G1 and G2 phases in the cell cycle wherein the cell growth inhibition is caused by inhibition of histone deacetylase.

iv) **Cold stress**

Stress induced in a cell by lowering the temperature at which a cell or organism optimally grows at. Many of the effects of cold stress are similar to the effects of heat shock on cells, including induction of a set of gene products, down regulation of other gene products, and an overall alteration of chromatin structure.

v) **Magnetic field**

Emission or propagation of electromagnetic energy. Magnetic fields act to enhance the genotoxic effect of spontaneously produced free radicals, possibly by effecting the lifetime of the radicals, and thus may act to alter chromatin structure by free radical damage.

vi) **Aflatoxin B1**

Aflatoxin B1 is a member of a group of closely related toxic metabolites that are designated mycotoxins. They are produced by Aspergillus flavus and A. parasiticus. Members of the group include aflatoxin B1, aflatoxin B2, aflatoxin G1, aflatoxin G2, aflatoxin M1, and aflatoxin M2. The chemical structure of Aflatoxin B1 is 6aR-cis-2,3,6 aalpha,9aalpha-Tetrahydro-4-methoxycyclopenta(c)furo(3',2':4,5)furo(2,3-h)(1)benzo-pyran -1,11-dione. It is a potent hepatotoxic and hepatocarcinogenic mycotoxin produced by the Aspergillus flavus group of fungi. It is also mutagenic, teratogenic, and causes immunosuppression in animals. It is found as a contaminant in peanuts, cottonseed meal, corn, and other grains. The mycotoxin requires epoxidation to aflatoxin B1 2,3-oxide for activation. Microsomal monooxygenases biotransform the toxin to the less toxic metabolites aflatoxin M1 and Q1. Aflatoxin is know to effect DNA conformation. Since Aflatoxin can act as a mutagen it can also be considered to mediate DNA damage.

vii) **Actinomycin D**

Actinomycin D (AMD) is disclosed in German patent No. 1,172,680 and is a chromopeptide antibiotic whose potent activity in several tumors, including Wilm's tumor, gestational choriocarcinoma and Kaposi's sarcoma, has been reported. AMD at submicromolar concentrations strongly inhibits DNA-dependent RNA synthesis and, to a lesser extent, DNA synthesis. Its interaction with DNA has been extensively studied, and the details of the mechanism of binding to DNA has been proposed (Reich, 1963; Muller and Crothers, 1968; Sobell and Jain, 1972). It has been assumed

that the cytotoxicity of AMD is due to its inhibition of RNA polymerase following the intercalative binding to DNA. It is quite possible, however, that the distortions in helical DNA resulting from the strong noncovalent association with AMD may not be solely responsible for the observed biological effects. Nakazawa *et al*, (1981) suggest that an intermediate free-radical form of AMD may be an active form that causes DNA damage and cell death, thus AMD can also be considered a DNA damaging agent.

C. Treatments Effecting the Recombination Machinery

i) 3-methoxybenzamide

3-methoxybenzamide (3-MB) is an inhibitor of poly(ADP-ribose) polymerase, an enzyme which catalyzes the incorporation of ADP-ribose groups of NAD+ into a homopolymer of repeating ADP-ribose units (EC 2.4.2.30). Because histones can also be ADP-riboseylated, 3-MB may be considered a modifier of chromatin structure.

ii) Caffeine

A methylxanthine naturally occurring in some beverages and also used as a pharmacological agent. Caffeine's most notable pharmacological effect is as a central nervous system stimulant, increasing alertness and producing agitation. It also relaxes smooth muscle, stimulates cardiac muscle, stimulates diuresis, and appears to be useful in the treatment of some types of headache. Several cellular actions of caffeine have been observed, but it is not entirely clear how each contributes to its pharmacological profile. Among the most important are inhibition of cyclic nucleotide phosphodiesterases, antagonism of adenosine receptors, and modulation of intracellular calcium handling.

VI. Plants

As used herein the term "progeny" denotes the offspring of any generation of a parent plant prepared in accordance with the instant invention. "Crossing" a plant to provide a plant line having one or more added transgenes relative to a starting plant

line, as disclosed herein, is defined as the techniques that result in a transgene of the invention being introduced into a plant line by mating a starting line to a donor plant line that comprises a transgene of the invention. To achieve this one would, generally, perform the following steps:

(a) plant seeds of the first (starting line) and second (donor plant line that comprises a transgene of the invention) parent plants;

(b) grow the seeds of the first and second parent plants into plants that bear flowers;

(c) pollinate the female flower of one parent plant with the pollen of the other parent; and

(d) harvest seeds produced on the parent plant bearing the female flower.

Backcross conversion is herein defined as the process including the steps of:

(a) crossing a plant of a first genotype containing a desired trait, gene, DNA sequence or element to a plant of a second genotype lacking said desired trait, gene, DNA sequence or element;

(b) selecting one or more progeny plant containing the desired gene, DNA sequence or element;

(c) crossing the progeny plant to a plant of the second parental genotype; and

(d) repeating steps (b) and (c) for the purpose of transferring said desired gene, DNA sequence or element from a plant of a first parental genotype to a plant of a second parental genotype.

"Introgression" means the breeding or crossing of a DNA element into a plant genotype as the result of the process of backcross conversion. A plant genotype into which a DNA sequence has been introgressed may be referred to as a backcross converted genotype, line, inbred, or hybrid. Similarly a plant genotype lacking said desired DNA sequence may be referred to as an unconverted genotype, line, inbred, or hybrid.

"Gymnosperms" means a member of a group of vascular plants whose seeds are not enclosed by a ripened ovary (fruit). Gymnosperms are distinguished from the other major group of seed plants, the angiosperms, whose seeds are surrounded by an ovary wall. The seeds of many gymnosperms (literally, "naked seed") are typically borne in cones and are not visible. Taxonomists now recognize four distinct divisions of extant gymnospermous plants (*Coniferophyta, Cycadophyta, Ginkgophyta,* and *Gnetophyta*).

"Angiosperm" means any member of the more than 235,000 species of flowering plants (phylum *Anthophyta*) having roots, stems, leaves, and well-developed conductive tissues (xylem and phloem). Angiosperms are often differentiated from gymnosperms by their production of seeds within a closed chamber (the ovary). The *Anthophyta* are divided into the monocotyledones or monocots and dicotyledones or dicots.

"Monocot" means a large class of plants of the *Anthophyta* phylum which is characterized by having a single cotyledon (seed leaf). Included are maize, rice, wheat, barley, sorghum, oat, and sugarcane.

"Dicot" means a large class of plants of the *Anthophyta* phylum which is characterized by having two cotyledons (seed leafs). Included are cotton, tobacco, tomato, soybean, sunflower, oil seed rape (canola), alfalfa, potato, strawberry, onion, broccoli, *Arabidopsis*, pepper, and citrus.

"*Brassica*" is a large genus of often edible, cruciferous plants including the large species *Brassica oleracea*. Included are cabbage, cauliflower, broccoli, Brussel sprout, kale, and collard green (all are the same species), turnip (*B. napa*), rutabaga (*B. napobrassica*), rapeseed (*B. napus*), and mustard (*B. alba, B. junica, and B. nigra*).

"*Arabidopsis*" is a genus of flowering plants found in north temperate regions. The species *Arabidopsis thaliana* is used for experiments in classical plant genetics as well as molecular genetic studies in plant physiology, biochemistry, and development.

VII. Tetrad Analysis

The *quartet* (qrt 1) mutation causes the four products of pollen mother cell meiosis in *Arabidopsis* to remain attached. When used to pollinate a flower, one tetrad can result in the formation of four seeds, and the plants from these seeds can be analyzed genetically using tetrad analysis (Copenhaver, 1998).

With unordered tetrads, such as those produced by *S. cerevisiae* or *Arabidopsis*, genetic mapping using tetrad analysis requires that two markers be scored simultaneously (Whitehouse 1950). Tetrads fall into different classes depending on whether the markers are in a parental (non-recombinant) or nonparental (recombinant) configuration (Copenhaver *et al.*, 1998). A tetrad with only non-recombinant members is referred to as a parental ditype (PD); one with only recombinant members as a nonparental ditype (NPD); and a tetrad with two recombinant and two non-recombinant members as a tetratype (TT) (Perkins 1953). If two genetic loci are on different chromosomes, and thus assort independently, the frequency of tetratype (crossover products) versus parental or nonparental assortment ditype (non-crossover products) depends on the frequency of crossover between each of the two loci and their respective centromeres. The genetic distance (in centimorgans, cM) between any two markers centromeres is defined by the function [(1/2)TT]/100 (Mortimer *et al.*, 1981).

The recessive *qrt1* mutation makes it possible to perform tetrad analysis in *Arabidopsis* by causing the four products of meiosis to remain attached (Preuss *et al.*, 1994; and Smythe 1994; both incorporated herein by reference). As previously shown, within each tetrad, genetic loci segregate in a 2:2 ratio. Individual tetrads can be manipulated onto flowers with a fine brush (at a rate of 20 tetrads per hour), and in

30% of such crosses, four viable seeds can be obtained (Preuss *et al.*, 1994; Copenhaver, 1998).

VIII. Transformation of Plants

Methods and compositions for transforming a plant cell, or an entire plant with genetic constructs are further aspects of this disclosure. Two general approaches are used for introduction of new genetic information ("transformation") into cells. One approach is to introduce the new genetic information as part of another DNA molecule, referred to as a "vector," which can be maintained as an independent unit (an episome) apart from the chromosomal DNA molecule(s). Episomal vectors contain all the necessary DNA sequence elements required for DNA replication and maintenance of the vector within the cell. Many episomal vectors are available for use in bacterial cells (for example, see Maniatis *et al.*, 1982). However, only a few episomal vectors that function in higher eukaryotic cells have been developed. The available higher eukaryotic episomal vectors are based on naturally occurring viruses and most function only in mammalian cells (Willard, 1997). In higher plant systems the only known double-stranded DNA viruses that replicate through a double-stranded intermediate upon which an episomal vector could be based is the gemini virus, although the gemini virus is limited to an approximately 800 bp insert. Although an episomal plant vector based on the Cauliflower Mosaic Virus has been developed, its capacity to carry new genetic information also is limited (Brisson *et al.*, 1984).

The other general method of genetic transformation involves integration of introduced DNA sequences into the recipient cell's chromosomes, permitting the new information to be replicated and partitioned to the cell's progeny as a part of the natural chromosomes. The most common form of integrative transformation is called "transfection" and is frequently used in mammalian cell culture systems. Transfection involves introduction of relatively large quantities of deproteinized DNA into cells. The introduced DNA usually is broken and joined together in various combinations before it is integrated at random sites into the cell's chromosome (see, for example Wigler *et al.*, 1977). Common problems with this procedure are the rearrangement of

introduced DNA sequences and unpredictable levels of expression due to the location of the transgene in the genome or so called "position effect variation" (Shingo *et al.*, 1986). Further, unlike episomal DNA, integrated DNA cannot normally be precisely removed. A more refined form of integrative transformation can be achieved by exploiting naturally occurring viruses that integrate into the host's chromosomes as part of their life cycle, such as retroviruses (see Cepko *et al.*, 1984). In mouse, homologous integration has recently become common, although it is significantly more difficult to use in plants (Lam *et al.* 1996).

The most common genetic transformation method used in higher plants is based on the transfer of bacterial DNA into plant chromosomes that occurs during infection by the phytopathogenic soil bacterium *Agrobacterium* (see Nester *et al.*, 1984). By substituting genes of interest for the naturally transferred bacterial sequences (called T-DNA), investigators have been able to introduce new DNA into plant cells. However, even this more "refined" integrative transformation system is limited in three major ways. First, DNA sequences introduced into plant cells using the *Agrobacterium* T-DNA system are frequently rearranged (Jones *et al.*, 1987). Second, the expression of the introduced DNA sequences varies between individual transformants (see Jones *et al.*, 1985). This variability is presumably caused by rearranged sequences and the influence of surrounding sequences in the plant chromosome (*i.e.*, position effects), as well as methylation of the transgene. A third drawback of the *Agrobacterium T-DNA* system is the reliance on a "gene addition" mechanism: the new genetic information is added to the genome (*i.e.*, all the genetic information a cell possesses) but does not replace information already present in the genome.

Means for transforming bacteria and yeast cells are well known in the art. Typically, means of transformation are similar to those well known means used to transform other bacteria or yeast such as *E. coli* or *Saccharomyces cerevisiae*. Methods for DNA transformation of plant cells include *Agrobacterium*-mediated plant transformation, protoplast transformation, gene transfer into pollen, injection into

reproductive organs, injection into immature embryos and particle bombardment. Each of these methods has distinct advantages and disadvantages. Thus, one particular method of introducing genes into a particular plant strain may not necessarily be the most effective for another plant strain, but it is well known in the art which methods are useful for a particular plant strain.

There are many methods for introducing transforming DNA segments into cells, but not all are suitable for delivering DNA to plant cells. Suitable methods are believed to include virtually any method by which DNA can be introduced into a cell, such as by *Agrobacterium* infection, direct delivery of DNA such as, for example, by PEG-mediated transformation of protoplasts (Omirulleh *et al.*, 1993), by desiccation/inhibition-mediated DNA uptake, by electroporation, by agitation with silicon carbide fibers, by acceleration of DNA coated particles, *etc.* In certain embodiments, acceleration methods are preferred and include, for example, microprojectile bombardment and the like.

Technology for introduction of DNA into cells is well-known to those of skill in the art. Four general methods for delivering a gene into cells have been described: (1) chemical methods (Graham *et al.*, 1973; Zatloukal *et al.*, 1992); (2) physical methods such as microinjection (Capecchi, 1980), electroporation (Wong *et al.*, 1982; Fromm *et al.*, 1985; U. S. Patent No. 5,384,253) and the gene gun (Johnston *et al.*, 1994; Fynan *et al.*, 1993); (3) viral vectors (Clapp 1993; Lu *et al.*, 1993; Eglitis *et al.*, 1988a; 1988b); and (4) receptor-mediated mechanisms (Curiel *et al.*, 1991; 1992; Wagner *et al.*, 1992).

A. **Electroporation**

The application of brief, high-voltage electric pulses to a variety of animal and plant cells leads to the formation of nanometer-sized pores in the plasma membrane. DNA is taken directly into the cell cytoplasm either through these pores or as a consequence of the redistribution of membrane components that accompanies closure of the pores. Electroporation can be extremely efficient and can be used both for transient expression of cloned genes and for establishment of cell lines that carry integrated copies of the gene of interest. Electroporation, in contrast to calcium phosphate-mediated transfection and protoplast fusion, frequently gives rise to cell lines that carry one, or at most a few, integrated copies of the foreign DNA.

The introduction of DNA by means of electroporation, is well-known to those of skill in the art. In this method, certain cell wall-degrading enzymes, such as pectin-degrading enzymes, are employed to render the target recipient cells more susceptible to transformation by electroporation than untreated cells. Alternatively, recipient cells are made more susceptible to transformation, by mechanical wounding. To effect transformation by electroporation one may employ either friable tissues such as a suspension culture of cells, or embryogenic callus, or alternatively, one may transform immature embryos or other organized tissues directly. One would partially degrade the cell walls of the chosen cells by exposing them to pectin-degrading enzymes (pectolyases) or mechanically wounding in a controlled manner. Such cells would then be recipient to DNA transfer by electroporation, which may be carried out at this stage, and transformed cells then identified by a suitable selection or screening protocol dependent on the nature of the newly incorporated DNA.

B. **Microprojectile Bombardment**

A further advantageous method for delivering transforming DNA segments to plant cells is microprojectile bombardment. In this method, particles may be coated with nucleic acids and delivered into cells by a propelling force. Exemplary particles include those comprised of tungsten, gold, platinum, and the like.

An advantage of microprojectile bombardment, in addition to it being an effective means of reproducibly stably transforming monocots, is that neither the isolation of protoplasts (Cristou *et al.*, 1988) nor the susceptibility to *Agrobacterium* infection is required. An illustrative embodiment of a method for delivering DNA into maize cells by acceleration is a Biolistics Particle Delivery System, which can be used to propel particles coated with DNA or cells through a screen, such as a stainless steel or Nytex screen, onto a filter surface covered with plant cells cultured in suspension. The screen disperses the particles so that they are not delivered to the recipient cells in large aggregates. It is believed that a screen intervening between the projectile apparatus and the cells to be bombarded reduces the size of projectiles aggregate and may contribute to a higher frequency of transformation by reducing damage inflicted on the recipient cells by projectiles that are too large.

For the bombardment, cells in suspension are preferably concentrated on filters or solid culture medium. Alternatively, immature embryos or other target cells may be arranged on solid culture medium. The cells to be bombarded are positioned at an appropriate distance below the macroprojectile stopping plate. If desired, one or more screens also are positioned between the acceleration device and the cells to be bombarded. Through the use of techniques set forth herein one may obtain up to 1000 or more foci of cells transiently expressing a marker gene. The number of cells in a focus which express the exogenous gene product 48 hours post-bombardment often range from 1 to 10 and average 1 to 3.

In bombardment transformation, one may optimize the prebombardment culturing conditions and the bombardment parameters to yield the maximum numbers of stable transformants. Both the physical and biological parameters for bombardment are important in this technology. Physical factors are those that involve manipulating the DNA/microprojectile precipitate or those that affect the flight and velocity of either the macro- or microprojectiles. Biological factors include all steps involved in manipulation of cells before and immediately after bombardment, the osmotic adjustment of target cells to help alleviate the trauma associated with

bombardment, and also the nature of the transforming DNA, such as linearized DNA or intact supercoiled plasmids. It is believed that pre-bombardment manipulations are especially important for successful transformation of immature embryos.

Accordingly, it is contemplated that one may wish to adjust various of the bombardment parameters in small scale studies to fully optimize the conditions. One may particularly wish to adjust physical parameters such as gap distance, flight distance, tissue distance, and helium pressure. One also may minimize the trauma reduction factors (TRFs) by modifying conditions which influence the physiological state of the recipient cells and which may therefore influence transformation and integration efficiencies. For example, the osmotic state, tissue hydration and the subculture stage or cell cycle of the recipient cells may be adjusted for optimum transformation. The execution of other routine adjustments will be known to those of skill in the art in light of the present disclosure.

C. *Agrobacterium*-Mediated Transfer

Agrobacterium-mediated transfer is a widely applicable system for introducing genes into plant cells because the DNA can be introduced into whole plant tissues, thereby bypassing the need for regeneration of an intact plant from a protoplast. The use of *Agrobacterium*-mediated plant integrating vectors to introduce DNA into plant cells is well known in the art. See, for example, the methods described (Fraley *et al.*, 1985; Rogers *et al.*, 1987). Using conventional transformation vectors, chromosomal integration is required for stable inheritance of the foreign DNA. However, the vector described herein may be used for transformation with or without integration, as the centromere function required for stable inheritance is encoded within the PLAC. In particular embodiments., transformation events in which the PLAC is not chromosomally integrated may be preferred, in that problems with site-specific variations in expression and insertional mutagenesis may be avoided.

The integration of the Ti-DNA is a relatively precise process resulting in few rearrangements. The region of DNA to be transferred is defined by the border

sequences, and intervening DNA is usually inserted into the plant genome as described (Spielmann *et al.*, 1986; Jorgensen *et al.*, 1987). Modern *Agrobacterium* transformation vectors are capable of replication in *E. coli* as well as *Agrobacterium*, allowing for convenient manipulations as described (Klee *et al.*, 1985). Moreover, recent technological advances in vectors for *Agrobacterium*-mediated gene transfer have improved the arrangement of genes and restriction sites in the vectors to facilitate construction of vectors capable of expressing various polypeptide coding genes. The vectors described (Rogers *et al.*, 1987), have convenient multi-linker regions flanked by a promoter and a polyadenylation site for direct expression of inserted polypeptide coding genes and are suitable for present purposes. In addition, *Agrobacterium* containing both armed and disarmed Ti genes can be used for the transformations. In those plant strains where *Agrobacterium*-mediated transformation is efficient, it is the method of choice because of the facile and defined nature of the gene transfer.

Agrobacterium-mediated transformation of leaf disks and other tissues such as cotyledons and hypocotyls appears to be limited to plants that *Agrobacterium* naturally infects. *Agrobacterium*-mediated transformation is most efficient in dicotyledonous plants. Few monocots appear to be natural hosts for *Agrobacterium*, although transgenic plants have been produced in asparagus and more significantly in maize using *Agrobacterium* vectors as described (Bytebier *et al.*, 1987; U.S. Patent No. 5,591,616, specifically incorporated herein by reference). Therefore, commercially important cereal grains such as rice, corn, and wheat must usually be transformed using alternative methods. However, as mentioned above, the transformation of asparagus using *Agrobacterium* also can be achieved (see, for example, Bytebier *et al.*, 1987).

A transgenic plant formed using *Agrobacterium* transformation methods typically contains a single gene on one chromosome. Such transgenic plants can be referred to as being hemizygous for the added gene. A more accurate name for such a

plant is an independent segregant, because each transformed plant represents a unique T-DNA integration event.

More preferred is a transgenic plant that is homozygous for the added foreign DNA, *i.e.*, a transgenic plant that contains two copies of a transgene, one gene at the same locus on each chromosome of a chromosome pair. A homozygous transgenic plant can be obtained by sexually mating (selfing) an independent segregant transgenic plant that contains a single added transgene, germinating some of the seed produced and analyzing the resulting plants produced for enhanced activity relative to a control (native, non-transgenic) or an independent segregant transgenic plant.

D. Other Transformation Methods

Transformation of plant protoplasts can be achieved using methods based on calcium phosphate precipitation, polyethylene glycol treatment, electroporation, and combinations of these treatments (see, *e.g.*, Potrykus *et al.*, 1985; Lorz *et al.*, 1985; Fromm *et al.*, 1986; Uchimiya *et al.*, 1986; Callis *et al.*, 1987; Marcotte *et al.*, 1988).

Application of these systems to different plant strains for the purpose of making transgenic plants depends upon the ability to regenerate that particular plant strain from protoplasts. Illustrative methods for the regeneration of cereals from protoplasts are described (Fujimura *et al.*, 1985; Toriyama *et al.*, 1986; Yamada *et al.*, 1986; Abdullah *et al.*, 1986).

To transform plant strains that cannot be successfully regenerated from protoplasts, other ways to introduce DNA into intact cells or tissues can be utilized. For example, regeneration of cereals from immature embryos or explants can be effected as described (Vasil 1988). In addition, "particle gun" or high-velocity microprojectile technology can be utilized (Vasil 1992).

Using that latter technology, DNA is carried through the cell wall and into the cytoplasm on the surface of small metal particles as described (Klein *et al.*, 1987;

Klein *et al.*, 1988; McCabe *et al.*, 1988). The metal particles penetrate through several layers of cells and thus allow the transformation of cells within tissue explants.

IX. Definitions

"Transformation" or "Transfection" means the acquisition in cells of new DNA sequences through incorporation of added DNA. This is the process by which naked DNA, DNA coated with protein, or whole artificial chromosomes are introduced into a cell, resulting in a heritable change.

"Gene" means a DNA sequence that contains information for construction of a polypeptide or protein, and includes 5' and 3' ends. This also includes genes which encode only RNA products such as tRNA or rRNA genes.

As used herein, "eukaryote" refers to living organisms whose cells contain nuclei. A eukaryote may be distinguished from a "prokaryote" which is an organism which lacks nuclei. Prokaryotes and eukaryotes differ fundamentally in the way their genetic information is organized, as well as their patterns of RNA and protein synthesis.

The term "lower eukaryote" means a eukaryote characterized by a comparatively simple physiology and composition, and most often unicellularity. Examples of lower eukaryotes include flagellates, ciliates, and yeast.

In contrast, the term "higher eukaryote" means a multicellular eukaryote, typically characterized by its greater complex physiological mechanisms and relatively large size. Generally, complex organisms such as plants and animals are included in this category.

As used herein, the term "plant" includes plant cells, plant protoplasts, plant calli, and the like, as well as whole plants regenerated therefrom.

As used herein, "heterologous gene" or "foreign gene" is a structural gene that is foreign, *i.e.*, originating from a donor different from the host or a chemically synthesized gene, and can include a donor of a different species from the host. The heterologous gene codes for a polypeptide or RNA ordinarily not produced by the organism susceptible to transformation by the expression vehicle. Another type of "heterologous gene" is an altered gene from the host itself, or an unaltered gene which is present in one or more extra copies. One example of such an altered gene useful in the present invention is a mutant gene which encodes a herbicide-resistant form of a normally occurring enzyme.

"Host" means any organism that is the recipient of a replicable plasmid, or expression vector comprising an artificial chromosome. Ideally, host strains used for cloning experiments should be free of any restriction enzyme activity that might degrade the foreign DNA used. Preferred examples of host cells for cloning, useful in the present invention, are bacteria such as *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas*, *Streptomyces*, *Salmonella*, and yeast cells such as *S. cerevisiae*. Host cells which can be targeted for expression of an artificial chromosome may be plant cells of any source and specifically include *Arabidopsis*, maize, rice, sugarcane, sorghum, barley, soybeans, tobacco, wheat, tomato, potato, citrus, or any other agronomically or scientifically important species.

"Expression" means the process by which a structural gene produces an RNA molecule, typically termed messenger RNA (mRNA). The mRNA is typically, but not always, translated into polypeptide(s).

"Linker" means a DNA molecule, generally up to 50 or 60 nucleotides long and synthesized chemically, or cloned from other vectors. In a preferred embodiment, this fragment contains one, or preferably more than one, restriction enzyme site for a blunt-cutting enzyme and a staggered-cutting enzyme, such as *Bam*HI. One end of

the linker fragment is adapted to be ligatable to one end of the linear molecule and the other end is adapted to be ligatable to the other end of the linear molecule.

"Homologous recombination" means any process that generates new gene or chromosomal recombinations by means of crossing-over between similar sequences.

"Non-homologous recombination" means any process that creates new gene or chromosomal combinations by means of crossing over between DNA strands which are not similar in sequence.

"Stimulated recombination" means any process or treatment which increases the frequency or absolute number of recombination events which occur during mitosis, meiosis or transformation

"Backcross" means the crossing of an individual with one of its parents or a genetically equivalent organism.

"Introgression" means a process whereby a series of directed matings is used to move a trait, gene, or DNA segment from one genetic background, strain, or species into another genetic background, strain or species.

"Chromatin" means the substance of chromosomes including DNA, chromosomal proteins and chromosomal RNA.

"Recombination machinery" means the various cellular components such as enzymes, DNA substrate, chromosomal proteins and RNAs that participate in the recombination process.

"F1" means filial generation number 1.

"F2" means filial generation number 2.

As used herein, a "library" is a pool of random DNA fragments which are cloned. In principle, any gene can be isolated by screening the library with a specific hybridization probe (see, for example, Young *et al.*, 1977). Each library may contain the DNA of a given organism inserted as discrete restriction enzyme-generated fragments or as randomly sheered fragments into many thousands of plasmid vectors. For purposes of the present invention, *E. coli*, yeast, and *Salmonella* plasmids are particularly useful when the genome inserts come from other organisms.

"Hybridization" means the pairing of complementary RNA and DNA strands to produce an RNA-DNA hybrid, or alternatively, the pairing of two DNA single strands from genetically different or the same sources to produce a double-stranded DNA molecule.

The term "plasmid" or "cloning vector" as used herein refers to a closed covalently circular extrachromosomal DNA or linear DNA which is able to autonomously replicate in a host cell and which is normally nonessential to the survival of the cell. A wide variety of plasmids and other vectors are known and commonly used in the art (see, for example, Cohen *et al.*, U.S. Pat. No. 4,468,464, which discloses examples of DNA plasmids, and which is specifically incorporated herein by reference).

As used herein, a "probe" is any biochemical reagent (usually tagged in some way for ease of identification), used to identify or isolate a gene, a gene product, a DNA segment or a protein.

A "selectable marker" is a gene whose presence results in a clear phenotype, and most often a growth advantage for cells that contain the marker. This growth advantage may be present under standard conditions, altered conditions such as elevated temperature, or in the presence of certain chemicals such as herbicides or antibiotics. Use of selectable markers is described, for example, in Broach *et al.*

(1979). Examples of selectable markers include the thymidine kinase gene, the cellular adenine-phosphoribosyltransferase gene and the dihydrylfolate reductase gene, hygromycin phosphotransferase genes, the bar gene and neomycin phosphotransferase genes, among others. Preferred selectable markers in the present invention include genes whose expression confer antibiotic or herbicide resistance to the host cell, sufficient to enable the maintenance of a vector within the host cell, and which facilitate the manipulation of the plasmid into new host cells. Of particular interest in the present invention are proteins conferring cellular resistance to ampicillin, chloramphenicol, tetracycline, G-418, bialaphos, and glyphosate for example.

X. Examples

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skilled the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

EXAMPLE 1

Treatments for Increased Recombination

Various chemical and environmental treatments were examined for their ability to stimulate recombination in *Arabidopsis thaliana*. The treatments were used on pollen donors in crosses performed to create tetrad sets of plants (Copenhaver,

1998). These sets of tetrad plants were then used to perform tetrad analysis in order to assay recombination frequencies. F1 hybrid pollen donor plants were planted individually in 1 inch square pots and grown under 24 hr light in a growth room until flowering. Flowering plants were then dipped in one of the following solutions and watered with 50 ml of the same solution.

Table 1: Treatment Agents.

COMPOUND	SOURCE	CONCENTRATION RANGE
n-butyric acid	Sigma	from about 0.1mg/L to about 50 mg/L
UV exposure 350 nm	N/A	about 1 second to about 50 seconds
Methanesulfonic acid ethyl ester	Sigma	from about 0.01% to about 0.2%
5-aza-2'-deoxycytidine	Sigma	from about 0.1 mg/L to about 50 mg/L
heat shock	N/A	about 28-49 C for 5 min to continual
3-methoxybenzamide	Aldrich	from about 0.1 mM to about 10 mM
Zeocin	Invitrogen	from about 0.1 µg/ml to about 5 µg/ml
Methanesulfonic acid methyl ester	Sigma	from about 0.1 ppm to about 200 ppm
cis-diamminedichloro-platinum	Aldrich	about 0.1 µg/ml to about 60 µg/ml
Mitomycin C	Sigma	from about 0.1 mg/L to about 100 mg/L
n-nitroso-n-ethylurea	Sigma	from about 0.1 µM to about 200 µM
Trichostatin A	Sigma	from about 0.1 µM to about 30 µM
gamma radiation	N/A	about 0.1 kRads to about 20 kRads

Table 1 (Cont'd)

COMPOUND	SOURCE	CONCENTRATION RANGE
cold stress	N/A	about -10 to 15C for 1 min to continuous
sodium azide	Sigma	from about 0.01 mM to 10mM
magnetic field	N/A	about 1 to 20 Tesla for 1 h to continuous
Dimethylnitrosamine	Sigma	from about 1 µM to about 1 mM
Bleomycin	Sigma	from about 0.1 mg/L to about 30 mg/L
aflotoxin B1	Sigma	from about 8 µg/ml to about 800 µg/ml
8-methoxypsoralen	Sigma	From about 0.01 mM to about 50 mM
Cyclophosphamide	Sigma	From about .001 mg/L to about 500 mg/L
Hydroxyurea	Sigma	From about 1 mM to about 500 mM
Actinomycin D	Sigma	From about 0.0001% to about 0.1% solution
Diepoxybutane	Sigma	from about 0.001% to about 1.0% solution
Caffeine	Sigma	from about 0.01% to about 5.0% solution

Following treatment, plants were then returned to the growth room and grown under standard conditions for 2-5 days. Pollen was then collected from newly opened flowers and used to pollinate receptive stigmas as described in (Copenhaver, 1998). Then the pollen donor plants were again treated as described above and used in

another round of pollination. Pollen donor plants were typically subjected to 5-10 rounds of treatment and pollen collection.

Heat shock treatments were performed by placing the pot containing the pollen donor plants in a shallow dish filled with water (to prevent desiccation), and placing the plant-containing dish in an incubator of the appropriate temperature. UV exposure was performed by placing the pollen donor plants in a BioRad UV chamber and illuminating the plants at the appropriate wave length for varying amounts of time. Both the UV and heat shock plants were subjected to several rounds of treatment and pollen collection. Plants exposed to a gamma radiation source (Cobalt-60) were treated only once and then discarded to prevent the accumulation of deleterious chromosomal rearrangements.

DNA was prepared from the tetrad sets of plants resulting from the crosses described above. Tetrad analysis (see section VII, Copenhaver, 1998) was used to measure the frequency of recombination between several linked pairs of PCR-based molecular markers. The number of tetratypes (TT) observed between the markers in a group of 384 untreated tetrad sets was compared to groups of treated tetrad sets. The number of recombination events which was expected to occur in each set of treated plants was calculated by multiplying the observed frequency (22/384) of recombination events in the untreated sets by the number of tetrad sets in each treatment category. Deviation from the null hypothesis that the treated plants display the same level of recombination frequencies as untreated controls was tested using the X^2 test ((observed – expected)^2/expected). Several treatments were shown to have stimulated recombination frequencies by this criteria (Table 2).

Table 2

Treatment	Tetrads	Obs	Exp	(O-E) $^2/E=X^2$
n-butyric acid	43	11	2.5	28.9**
UV exposure 350 nm	57	12	3.2	24.2**
Methanesulfonic acid ethyl ester	10	5*	0.6	32.2**
5-aza-2'-deoxycytidine	68	16	3.9	37.5**
heat shock	23	7	1.3	25.0**
3-methoxybenzamide	44	8	2.5	12.1**
Zeocin	106	14	6.0	10.6**
Untreated	384	22	N/A	N/A

** indicates significant by X^2 (df=1)

EXAMPLE 2

Facilitation of Genetic Map Creation

The techniques or treatments which stimulate recombination can be used to facilitate the generation of genetic maps. Genetic maps are the cornerstone of numerous scientific endeavors. The techniques taught by this disclosure could facilitate the construction of genetic maps in two ways. First the resolution of a genetic map, and therefore its accuracy is determined in part by the number of recombination events which can be scored in the population used to create the map. Typically this hurdle is overcome by scoring a large number of individuals which can be expensive and time consuming. Thus any techniques which increase these events will be of great value. Second, one difficulty often encountered in the use of genetic maps is that the ratio between genetic distances (measured in centiMorgans) and physical distance (in base pairs) is not always constant from one region of a genome to another. It has been observed that this disparity can be due to variance in chromatin structure. Because some of the treatments we describe modify chromatin structure it is possible that they will allow the creation of genetic maps which more accurately reflect true physical distance. An example which specifically embodies the

use of techniques or treatments which stimulate recombination to aid in the creation of genetic maps is described below.

Two strains which differ at numerous polymorphic loci (aabbccdd.... vs. AABBCCDD....) are crossed to create F1 hybrid progeny. These F1 hybrid progeny are then collected and exposed to the chemical or physical treatments described in section V using the methods described in Example 1. These treated F1 progeny are then either selfed or crossed to a genotypically defined tester strain to produce an F2 mapping population. Numerous molecular, biochemical or visible markers are then scored in each member of the mapping population and their segregation patterns are collated and analyzed by standard mapping functions to produce a genetic. The calculated genetic distances between any of the markers scored is directly related to the number of crossover events between the same markers in the mapping population. Thus treatments which stimulate the frequency of recombination will increase the resolution of the map.

Variations of this strategy include using the same treatments in the creation of recombinant inbred maps. In this embodiment two strains which differ at numerous polymorphic loci (aabbccdd.... vs. AABBCCDD....) are crossed to create F1 hybrid progeny (aAbBcCdD....). These F1 hybrid progeny are then collected and exposed to the chemical or physical treatments described in section V using the methods described in Example 1. These treated F1 progeny are then selfed or crossed to a genotypically defined tester strain to produce an series of F2 individuals. Each F2 individual is then allowed to self or siblings are interbred. A single F3 individual from each F2 line is isolated and allowed to self or crossed to siblings. This single line decent is continued as far as the F8 generation (or further) resulting in a highly recombinant mapping set. In this strategy the individuals in each selling step could also be treated to further enhance recombination.

EXAMPLE 3

Facilitation of Map-Based Positional Cloning

It is also contemplated by the inventors that one could employ techniques or treatments which stimulate recombination to facilitate map based positional cloning. Map based positional cloning describes the strategy whereby genetic mapping information is used to localize a desired gene or locus so that it may then be physically isolated by standard molecular techniques. The resolution of this method relies on the number of recombination events close to the desired locus. Typically this hurdle is overcome by scoring a large number of individuals which can be expensive and time consuming. Thus any techniques which increase these events will be of great value. An example which specifically embodies the use of techniques or treatments which stimulate recombination to aid map based positional cloning is described below.

A strain containing a phenotypically distinct allele (act) of the desired locus is crossed to a second strain that is homozygous for a second phenotypically distinct allele (AA) of the same locus. F1 hybrid progeny (aA) are collected and exposed to the chemical or physical treatments described in section V using the methods described in Example 1. These treated F1 progeny are then either selfed or crossed to a genotypically defined tester strain to produce an F2 population. The F2 population is then assayed to identify individuals with crossover events that occurred in the vicinity of the desired locus (informative recombinants) by scoring the segregation of molecular, biochemical or visible markers and comparing them to the phenotypic segregation of the locus in question. The ability to precisely define the physical location of a locus by this method is directly related to the number of informative recombinants produced in the creation of the F2 population. Therefor increasing the frequency of recombination during meiosis in the F1 parents will result in improved map based positional cloning.

EXAMPLE 4

Facilitation of Genetic Introgression

It is also contemplated by the inventors that one could employ techniques or treatments which stimulate recombination to facilitate introgression. Introgression describes a breeding technique whereby a desired trait is transferred into one strain (A) from another (B), the trait is then isolated in the genetic background of the desired strain (A) by a series of backcrosses to the same strain (A). The number of backcrosses required to isolate the desired trait in the desired genetic background is dependent on the frequency of recombination in each backcross. Typically numerous backcrosses must be performed which can be expensive and time consuming. Thus, any techniques which increase recombination frequencies will be of great value.

Backcrossing transfers a specific desirable trait from one source to an inbred or other plant that lacks that trait. This can be accomplished, for example, by first crossing a superior inbred (A) (recurrent parent) to a donor inbred (non-recurrent parent), which carries the appropriate gene(s) for the trait in question, for example, a construct prepared in accordance with the current invention. The progeny of this cross first are selected in the resultant progeny for the desired trait to be transferred from the non-recurrent parent, then the selected progeny are mated back to the superior recurrent parent (A). After five or more backcross generations with selection for the desired trait, the progeny are hemizygous for loci controlling the characteristic being transferred, but are like the superior parent for most or almost all other genes. The last backcross generation would be selfed to give progeny which are pure breeding for the gene(s) being transferred, *i.e.* one or more transformation events.

Therefore, through a series a breeding manipulations, a selected transgene may be moved from one line into an entirely different line without the need for further recombinant manipulation. Transgenes are valuable in that they typically behave genetically as any other gene and can be manipulated by breeding techniques in a manner identical to any other corn gene. Therefore, one may produce inbred plants

which are true breeding for one or more transgenes. By crossing different inbred plants, one may produce a large number of different hybrids with different combinations of transgenes. In this way, plants may be produced which have the desirable agronomic properties frequently associated with hybrids ("hybrid vigor"), as well as the desirable characteristics imparted by one or more transgene(s). An example which specifically embodies the use of techniques or treatments which stimulate recombination to aid introgression is described below.

A strain containing a desirable genetic background (XXYYWWZZ...) is crossed to a strain containing a desirable trait(s) (xxyywwzz, where the y allele is desired) with the intention of moving the trait in strain B into the genetic background of strain A. F1 progeny (XxYyWwZz) produced by this cross are collected and exposed to the chemical or physical treatments described in section V using the methods described in Example 1. These treated F1 progeny are then backcrossed to stain A to produce BC1 (backcross 1) progeny. Selection for the desired trait is applied to this generation (note, if homozygosity is required for detection of the desired trait then a selling or sibling-cross step must be included). Those BC1 individuals displaying the desired trait are collected and may or may not be treated again and backcrossed to strain A to produce a BC2 progeny. This backcrossing with selection process is continued as far as the BC8 (or more) generation until the desired genotype (AAbbCCDD....) is produced. The ability to precisely isolate the desired trait by this method is directly related to the number of recombination events occurring during each backcross generation. Therefore increasing the frequency of recombination during meiosis in each BC parent will result in improved introgression.

EXAMPLE 5

Facilitation of Genetic Transformation

It is also contemplated by the inventors that one could employ techniques or treatments which stimulate recombination to facilitate transformation. Transformation describes a number of molecular biological techniques whereby foreign or host DNA is introduced into the genome of a host cell by either homologous, non-homologous or site-specific recombination. The efficiency of these methods relies on recombination mechanisms and the efficiency of recombination determines the efficiency of the transformation procedure. Thus any techniques which increase recombination frequencies will be of great value. An example which specifically embodies the use of techniques or treatments which stimulate recombination to aid transformation techniques is described below.

A strain which is to be transformed is exposed to the chemical or physical treatments described in Section V using the methods described in Example 1. The treated strain is them transformed using one of a variety of standard transformation techniques including, but not limited to *Agrobacterium* mediated transformation, particle bombardment, liposome mediated transformation, PEG mediated cell fusion, virus mediate transformation or microinjection. The ability to efficiently transform a host genome is directly related to the efficiency of recombination. Therefore, increasing the frequency of recombination will result in improved transformation.

Variations on this strategy include treating the strain to be transformed at the same time the transforming DNA is introduced into the cell or after the transforming DNA is introduced into the cell.

All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations

may be applied to the compositions and methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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CLAIMS:

1. A method for increasing meiotic recombination in a plant comprising the steps of:

(a) crossing plant strain 1 to plant strain 2 to generate a heterozygous F1 hybrid;

(b) applying to the F1 hybrid a chemical or physical treatment that results in

DNA modification or damage;

(c) pollinate the treated F1 hybrid or pollinate with the treated F1 hybrid;

2. The method of claim 1, wherein the resulting seeds of said pollination are collected.

3. The method of claim 2, wherein the collected seeds are propagated to generate F2 progeny.

4. The method of claim 3, novel genetic recombinants in the F2 progeny are identified or screened for.

5. The method of claim 1, wherein the treated F1 hybrid is self-pollinated.

6. The method of claim 1, wherein the treated F1 hybrid is backcrossed a number of times resulting in introgression of a desired trait from strain 1 to strain 2.

7. The method of claim 1, wherein the method further comprises mapping of plant genes through increasing non-parental combinations of markers.

8. The method of claim 1, wherein strain 1 and a strain 2 are of different species.

9. The method of claim 1, wherein the plant is a gymnosperm.

10. The method of claim 1, wherein the plant is a crop.

11. The method of claim 1, wherein the plant is an angiosperm.

12. The method of claim 11, wherein the angiosperm is a monocot.

13. The method of claim 12, wherein the monocot is selected from the group consisting of maize, rice, wheat, barley, sorghum, oat, and sugarcane.

14. The method of claim 11, wherein the angiosperm is a dicot.

15. The method of claim 14, wherein the dicot is selected from the group consisting of cotton, pea, carrot, potato, cauliflower, broccoli, tobacco, tomato, soybean, sunflower, oil seed rape (canola), alfalfa, potato, strawberry, onion, broccoli, *Arabidopsis*, pepper, and citrus.

16. The method of claim 1, wherein the plant is a member of the *Brassica* genus.

17. The method of claim 16, wherein the *Brassica* genus is selected from the group consisting of cabbage, cauliflower, broccoli, Brussel sprout, kale, collard green, turnip, rutabaga, rapeseed and mustard.

18. The method of claim 1, wherein the plant is *Arabidopsis thaliana.*

19. The method of claim 1, wherein the treatment is U.V. exposure, methanesulfonic acid ethyl ester, 5-aza-2'-deoxycytidine, Zeocin, methanesulfonic acid methyl ester, cis-diamminedichloro-platinum, mitomycin C, n-nitroso-n-ethylurea, gamma radiation, sodium azide, dimethylnitrosamine, bleomycin, 8-methoxypsoralen, cyclophosphamide, hydroxyurea, or diepoxybutane.

20. The method of claim 19, wherein a combination of treatments are used.

21. The method of claim 19, wherein the treatment is U.V. exposure.

22. The method of claim 19, wherein the treatment is methanesulfonic acid ethyl ester.

23. The method of claim 19, wherein the treatment is 5-aza-2'-deoxycytidine.

24. The method of claim 19, wherein the treatment is Zeocin.

25. The method of claim 19, wherein the treatment is methanesulfonic acid methyl ester.

26. The method of claim 19, wherein the treatment is cis-diamminedichloro-platinum.

27. The method of claim 19, wherein the treatment is mitomycin C.

28. The method of claim 19, wherein the treatment is n-nitroso-n-ethylurea.

29. The method of claim 19, wherein the treatment is gamma radiation.

30. The method of claim 19, wherein the treatment is sodium azide.

31. The method of claim 19, wherein the treatment is dimethylnitrosamine.

32. The method of claim 19, wherein the treatment is bleomycin.

33. The method of claim 19, wherein the treatment is 8-methoxypsoralen.

34. The method of claim 19, wherein the treatment is cyclophosphamide.

35. The method of claim 19, wherein the treatment is hydroxyurea.

36. The method of claim 19, wherein the treatment is diepoxybutane.

37. A method for increasing meiotic recombination in a plant by the following:

(a) cross plant strain 1 to plant strain 2, generating a heterozygous F1 hybrid;

(b) applying to the F1 hybrid a chemical or physical treatment that results in

altering chromatin structure;

(c) pollinate the treated F1 hybrid or pollinate with the treated F1 hybrid;

38. The method of claim 37, wherein the resulting seeds of said pollination are collected.

39. The method of claim 38, wherein the collected seeds are propagated to generate F2 progeny.

40. The method of claim 39, novel genetic recombinants in the F2 progeny are identified or screened for.

41. The method of claim 37, wherein the treated F1 hybrid is self-pollinated.

42. The method of claim 37, wherein the treated F1 hybrid is backcrossed a number of times resulting in introgression of a desired trait from strain 1 to strain 2.

43. The method of claim 37, wherein the method further comprises mapping of plant genes through increasing non-parental combinations of markers.

44. The method of claim 37, wherein strain 1 and a strain 2 are of different species.

45. The method of claim 37, wherein the plant is a gymnosperm.

46. The method of claim 37, wherein the plant is a crop.

47. The method of claim 37, wherein the plant is an angiosperm.

48. The method of claim 47, wherein the angiosperm is a monocot.

49. The method of claim 48, wherein the monocot is selected from the group consisting of maize, rice, wheat, barley, sorghum, oat, and sugarcane.

50. The method of claim 47, wherein the angiosperm is a dicot.

51. The method of claim 50, wherein the dicot is selected from the group consisting of cotton, pea, carrot, potato, cauliflower, broccoli, tobacco, tomato, soybean, sunflower, oil seed rape (canola), alfalfa, potato, strawberry, onion, broccoli, *Arabidopsis*, pepper, and citrus.

52. The method of claim 37, wherein the plant is a member of the *Brassica* genus.

53. The method of claim 53, wherein the *Brassica* genus is selected from the group consisting of cabbage, cauliflower, broccoli, Brussel sprout, kale, collard green, turnip, rutabaga, rapeseed and mustard.

54. The method of claim 37, wherein the plant is *Arabidopsis thaliana.*

55. The method of claim 37, wherein the treatment is n-butyric acid, heat shock, trichostatin A, cold stress, magnetic field, aflatoxin B1, or actinomycin D.

56. The method of claim 37, wherein the treatment is a combination of compounds selected from the group consisting of n-butyric acid, heat shock, trichostatin A, cold stress, magnetic field, aflatoxin B1, and actinomycin D.

57. The method of claim 55, wherein the treatment is n-butyric acid.

58. The method of claim 55, wherein the treatment is heat shock.

59. The method of claim 55, wherein the treatment is trichostatin A.

60. The method of claim 55, wherein the treatment is cold stress.

61. The method of claim 55, wherein the treatment is magnetic field.

62. The method of claim 55, wherein the treatment is aflatoxin B1.

63. The method of claim 55, wherein the treatment is actinomycin D.

64. A method for increasing meiotic recombination in a plant by the following:

(a) cross plant strain 1 to plant strain 2, generating a heterozygous F1 hybrid;

(b) applying to the F1 hybrid a chemical or physical treatment that results in

altering the recombination machinery;

(c) pollinate the treated F1 hybrid or pollinate with the treated F1 hybrid;

65. The method of claim 64, wherein the resulting seeds of said pollination are collected.

66. The method of claim 65, wherein the collected seeds are propagated to generate F2 progeny.

67. The method of claim 66, wherein novel genetic recombinants in the F2 progeny are identified or screened for.

68. The method of claim 64, wherein the treated F1 hybrid is self-pollinated.

69. The method of claim 64, wherein the treated F1 hybrid is backcrossed a number of times resulting in introgression of a desired trait from strain 1 to strain 2.

70. The method of claim 64, wherein the method further comprises mapping of plant genes through increasing non-parental combinations of markers.

71. The method of claim 64, wherein strain 1 and a strain 2 are of different species.

72. The method of claim 64, wherein the plant is a gymnosperm.

73. The method of claim 64, wherein the plant is a crop.

74. The method of claim 64, wherein the plant is an angiosperm.

75. The method of claim 74, wherein the angiosperm is a monocot.

76. The method of claim 75, wherein the monocot is selected from the group consisting of maize, rice, wheat, barley, sorghum, oat, and sugarcane.

77. The method of claim 74, wherein the angiosperm is a dicot.

78. The method of claim 77, wherein the dicot is selected from the group consisting of cotton, tobacco, tomato, soybean, sunflower, oil seed rape (canola), alfalfa, potato, strawberry, onion, broccoli, *Arabidopsis*, pepper, and citrus.

79. The method of claim 64, wherein the plant is a member of the *Brassica* genus.

80. The method of claim 79, wherein the *Brassica* genus is selected from the group consisting of cabbage, cauliflower, broccoli, Brussel sprout, kale, collard green, turnip, rutabaga, rapeseed and mustard.

81. The method of claim 64, wherein the plant is *Arabidopsis thaliana.*

82. The method of claim 64, wherein the treatment is 3-methoxybenzamide

83. The method of claim 64, wherein the treatment is caffeine.

84. The method of claim 64, wherein a combination of chemical or physical treatments is used.

85. A method of increasing mitotic recombination in a plant by causing DNA modification or damage, altering chromatin structure, or altering recombination machinery of said plant by treating with a chemical or physical agent followed by propagating said plant vegetatively, or by producing seed, either through self or cross pollination.

86. A method of increasing transformation of a plant with a genetic element by causing DNA modification or damage, altering chromatin structure, or altering recombination machinery of said plant by treating with a chemical or physical agent followed by propagating said plant.

87. The method of claim 85, wherein the transformation and treating with a chemical or physical agent of said plant are simultaneous, the transformation is prior to treating with a chemical or physical agent of said plant, or the transformation follows treating with a chemical or physical agent of said plant.

88. The method of claim 85, wherein the transformation proceeds via non-homologous recombination.

89. The method of claim 85, wherein the transformation proceeds via homologous recombination.

90. The method of claim 85, wherein the transformation is via *Agrobacterium*, particle bombardment, liposome fusion, electroporation, microinjection, polyethylene and glycol mediated transformation.

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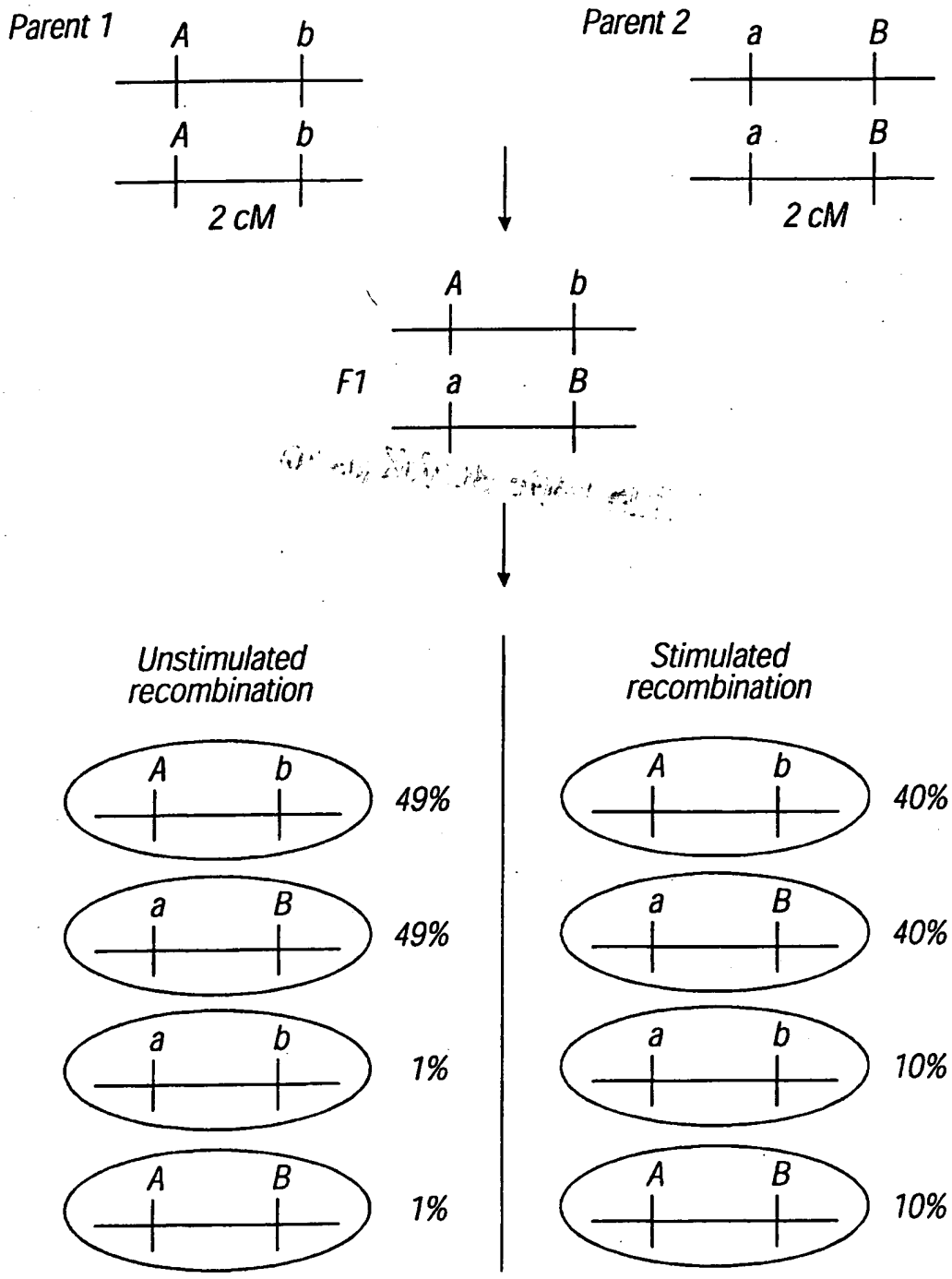
Parent 1
A
b
A
b
2 cM
Parent 2
a
B
a
B
2 cM
A
b
F1
a
B
Unstimulated recombination
A
b
49%
a
B
49%
a
b
1%
A
B
1%
Stimulated recombination
A
b
40%
a
B
40%
a
b
10%
A
B
10%

FIG. 1